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Role of acetic acid as antibacterial on *E.coli* isolated from young rabbits

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ABSTRACT

An investigation into the incidence of *E. coli* bacterial infections linked to outbreaks of rabbit diarrhea was carried out using a field survey. Ninety samples were recovered from diseased and freshly dead rabbits, suffering from diarrhea, from Kafrelsheikh governorate farms. Samples of fecal swabs and internal organs, including the liver, spleen, and intestinal contents, were aseptically collected, and *E. coli* was isolated and identified by traditional method. *E. coli* was typed serologically and tested for antimicrobial agents. *E. coli* infection incidence rate was (66.6%). Moreover, the serologically identified of seven *E. coli* isolates were three O91, two O128, one O17 and one O44. All bacterial isolates were highly sensitive to Ciprofloxacin, Amikacin, and Fosfomycin by 100%, while Tetracycline and Streptomycin resistance was present in 90% and 40% of the strains, respectively. The isolates were screened for presence of *cnf1* and *tsh* virulence genes, *qnrA* and *tetA(A)* antibiotic resistance genes. Six weeks-old thirty healthy rabbits were used, fecal swabs were taken to make sure that rabbits free from *E. coli*. Rabbits were divided into 5 groups (6 rabbits for each); Group 1 were kept as negative control (without infection), Group 2,3,4,5 were inoculated orally with 1 ml of culture (1×10^7 CFU/ml), Group 2 were kept as positive control (without treatment) and tested for the virulence by experimental examination, Group 3 were treated with ciprofloxacin 2 days after infection for 5 successive days, Group 4 treated with acetic acid 2 days after infection for 5 successive days, while Group 5 were treated with ciprofloxacin and acetic acid 2 days after infection for 5 successive days. All animals were kept for 21 days (period of observation) with daily examination for clinical signs, mortality rate and gross P.M. lesions in dead animals till the end of the observation period and trials of reisolation were conducted.

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Aim of the work:

The principal objective of the research was to find out the incidence of *E. coli* in young rabbits in the Egyptian governorate of Kafrelsheikh. The polymerase chain reaction (PCR) was also used to investigate the presence of certain virulence and antibiotic resistance genes in the isolated *E. coli* strains. Acetic acid alone and acetic acid with antibiotic were used as trials for treatment of artificially infected rabbits with *E. coli*.

INTRODUCTION

Since that rabbit meat has a high protein content, low fat content, and a delicious flavor, rabbits are crucial to the solution of the meat problem (Rashwan & Marai, 2000). The primary risk factor for gastrointestinal disorders is stress, and during the weaning phase of rabbits, diarrhea is one of the most common issues (Petrov et al. 2005). Serious issues with rabbit farming were encountered in Egypt, and illnesses that are costing this industry money are being closely monitored (Saif-Edin et al. 1994). Rabbit farming may be significantly impacted by diarrhea, a syndrome of digestive abnormalities in young rabbits that can result in secondary infections that lower immunity and increase newborn mortality (Chen et al. 2017). Currently, the primary cause of morbidity in growing rabbits is digestive disorders, which causes a sharp increase in the death rate in putting on weight rabbitries (Rosell et al. 2009).

Five days after weaning, The digesting enzymes in pancreatic tissue are not very active. because of interactions with other factors that might increase the chance of diarrhea after weaning (Hedemann and Jensen 2004). An imbalanced diet is one of the key risk factors for infections that cause enteritis, which is spread by the fecal-oral route (Newton et al. 2004). Some virulence-associated genes have been acquired by certain serotypes of this bacteria, allowing them to induce extraintestinal or intestinal illness. Enteric-pathogenic strains of *E. coli* are commonly referred to as diarrhea-genic strains, and their pathogenesis is linked to certain virulence characteristics that differ based on the pathotype (Xia et al. 2010). Provenance and Curtiss discovered the *tsh* gene recently, which codes for a temperature-sensitive

hemagglutinin (Provenance & Curtiss, 1994).

The most recent member of the autotransporter family IgA protease, which is present in many pathotypes of *Shigella spp.* And *Escherichia coli.*, is the *tsh* protein. A bacterial virulence factor linked to extraintestinal pathogenic *E. coli* strains is called Cytotoxic Necrotizing Factor 1 (CNF1). CNF1 toxin activity may exacerbate tissue damage and inflammation, according to studies (Schreiber et al. 2017). Due to the fact that rabbits were more prone to intestinal illnesses after they were weaned, prolonged use of antibiotics during therapy might only increase microbial resistance in agricultural animals. As a result, a number of substitutes have been developed, including organic acids, probiotics, and herbal extracts (Eiben et al. 2008). The intestinal mucosa in young rabbits is an essential barrier against antigenic attack and is involved in nutrition absorption and digesting (Gallois et al. 2005). The use of organic acids is crucial, Although it is true that there is a dearth of scientific evidence and frequently conflicting information about how they affect the microflora population, mucosal immunity, and growth performance in rabbits (Falcão-e-Cunha et al. 2007).

The organic acids play a direct action on the bacterial cell integrity (Maetrens et al. 2006). The antibacterial activity of acetic acid, which was traditionally diluted and used as vinegar, has probably been used for food preservation purposes longer than any other preservative due to its effects on food safety, wholesomeness, and quality. Because gut bacteria are kept from competing with the host for nutrients by organic acids, they are a reliable substitute for antibiotics in a rabbit's diet (Falcao-e-Cunha et al. 2007).

MATERIALS AND METHODS

Examined rabbits

Ninety samples in total were gathered from diseased & dead rabbits with a history of diarrhea. The samples were collected from different farms in Kafrelsheikh Governorate, clinically examined and put through a postmortem investigation in case of dead animals **OIE (2015)**.

Bacteriological examinations:

Isolation of *E. coli*.

E. coli was isolated from fecal sample and internal organs (spleen, intestine and liver) from diseased & freshly dead rabbits as stated by **Quinn et al. (2002)**, then *E. coli* isolates were recognized morphologically & biochemically based on **MacFaddin (2000)**. Diagnosing *E. coli* isolates with fast diagnostic sets of antisera (**DENKA SEIKEN Co., Japan**), **Kok et al. (1996)** identified the isolates based on serological analysis.

Antibiotic susceptibility testing

The disc diffusion method was used to 10 *E. coli* bacterial isolates using commercially available antibiotic discs, the following antibiotic discs were used: Ciprofloxacin (CIP) 5 ug, Amikacin (AK) 30 ug, Tetracycline (TE) 30ug, Fosfomycin (FO) 200 ug, Streptomycin (S) 10ug, Chloramphenicol (C) 30ug, Neomycin (N) 30 ug and Gentamicin (CN)10ug. In compliance with the guidelines provided by the National Committee for Clinical Laboratory Standards (**NCCLS, 2007**), then incubated at 37°C for a full day. The manufacturer's interpretation of the inhibitory zone was utilized to categorize isolates into groups that were either sensitive, intermediate, or resistant (**CLSI, 2018**).

The use of Polymerase chain reaction (PCR) to identify certain virulence and antibiotic resistance genes in *E. coli* isolates.

DNA extraction. Using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) and making certain adjustments based on the manufacturer's instructions, DNA was extracted from samples. In summary, 200 microliters of the

sample suspension were treated for 10 minutes at 56 degrees Celsius with 10 microliters of proteinase K and 200 microliters of lysis buffer. Following the incubation period, the lysate was mixed with 200 µl of 100% ethanol. The manufacturer's instructions were then followed while washing and centrifuging the sample. The elution buffer contained in the kit was 100 µl., which was used to elute the nucleic acid.

Oligonucleotide Primer. The primers utilized were provided by **Metabion (Germany)** and are mentioned in table (1).

PCR amplification. A twenty five µl reaction was conducted using one microliter of each primer at a concentration of 20 pmol, along with 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan) in addition to 5.5 µl of water., and 5 µl of DNA template. An Applied Biosystem 2720 heat cycler was used to carry out the process.

Analysis of the PCR Products.

The PCR products were electrophoresed on a 1.5% agarose gel (Appllichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. Fifteen microliters (µl) of each gel analysis item were placed within each gel slot. A generuler 100 bp ladder (Fermentas, Germany) was used to measure the fragment sizes. Biometra's gel documentation system, Alpha Innotech, was utilized to capture images of the gel, and data analysis was done using software.

Table 1. Primers sequences, target genes, amplicon sizes and cycling conditions.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	35 cycles of amplification)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
<i>tsh</i>	GGT GGT GCA CTG GAG TGG AGT CCA GCG TGA TAG TGG	620	94°C 5 min.	94°C 30 sec.	54°C 30 sec.	72°C 30 sec.	72°C 10 min.	(Delicato et al., 2003)
<i>cnf1</i>	TATATAGTCGTCAA- GATGGA CACTAAGCTTTACAA- TATTGAC	620	94°C 5 min.	94°C 30 sec.	63°C 45 sec.	72°C 30 sec.	72°C 7 min.	(Kadhum et al., 2008)
<i>tetA (A)</i>	GGTTCACTCGAACGAC- GTCA CTGTCCGACAAGTT- GCATGA	570	94°C 5 min.	94°C 30 sec.	50°C 40 sec.	72°C 45 sec.	72°C 10 min.	(Randall et al., 2004)
<i>qnrA</i>	ATTTCTCACGCCAGGAT- TTG GATCGG- CAAAGGTTAGGTCA	516	94°C 5 min.	94°C 30 sec.	55°C 40 sec.	72°C 45 sec.	72°C 10 min.	(Robicsek et al., 2006)

Experimental design:-

Preparation of *E. coli* infective dose :

For experimental infection, 30 healthy rabbits of 6 weeks-old were used, the bacterial isolate was adjusted by normal saline to a concentration of 1×10^7 CFU/ml (Skřivanová and Marounek, 2007) McFarland and used for this experiment. The rabbits were housed in cages and monitored for a week in order to assess their adaptability, fecal swabs were taken to make sure that rabbits free from *E. coli*, then divided into 5 groups (6 rabbits) : Group 1 were kept as control negative (without infection), Group 2 were inoculated orally with 1 ml of culture (1×10^7 CFU/ml) kept as positive control (without treatment), Group 3 were inoculated orally with 1 ml of culture (1×10^7 CFU/ml) and treated with ciprofloxacin two days following infection for five days in a row, Group 4 were inoculated orally with 1 ml of culture (1×10^7 CFU/ml) and treated with acetic acid Brand Chemicals (60%) 1ml /litre in drinking water 2 days after infection for 5 successive days, while Group 5 were inoculated

orally with 1 ml of culture (1×10^7 CFU/ml) and treated with ciprofloxacin and acetic acid 1ml /litre in drinking water 2 days after infection for 5 successive days. For a duration of 21 days, all animals were kept under observation. During this time, reisolation trials were carried out and daily checks were made for clinical symptoms, the mortality rate, and gross P.M. lesions in dead animals

RESULTS

Incidence of *E. coli* infection in rabbits:

A total of 90 rabbit samples were recovered from diseased & freshly dead rabbits from Kafrelsheikh governorate farms. Samples of fecal swabs & internal organs (liver, spleen and intestinal contents), yielded 60 *E. coli* positive samples with percent of 66.6 as shown in Table (2).

Table 2. Incidence of *E.coli*:

Samples	No. of +ve samples	Percentage %
90	60	66.6

Table 3. Identification of *E. coli* via serology

<i>E. coli</i> Serotype	No. of Strains
O91	three
O17	one
O128	two
O44	One
Total	seven

Table 4. Antimicrobial susceptibility test for *E.coli* isolates (no=10)

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No.	%	No.	%	No.	%
Ciprofloxacin (CIP)	10	100	-	-	-	-
Amikacin (AK)	10	100	-	-	-	-
Fosfomicin (FO)	10	100	-	-	-	-
Neomycin (N)	9	90	-	-	1	10
Gentamicin (CN)	9	90	-	-	1	10
Chloramphenicol (C)	9	90	-	-	1	10
Streptomycin (S)	4	40	2	20	4	40
Tetracycline (TE)	-	-	1	10	9	90

Table 5. Incidence of *E.coli* some virulence genes and antibiotic resistance genes in some isolates

No.of isolates	<i>cnf1</i>	<i>tsh</i>	<i>qnrA</i>	<i>tetA(A)</i>
1(O91)	-	+	+	+
2(O17)	+	+	+	-
3(O128)	-	+	+	+
4(O91)	-	+	+	-
5(O91)	+	+	+	+
6(O44)	-	-	+	+
7(O128)	-	+	+	+
Total%	2(28.5%)	6(85.7%)	7(100%)	5(71.4%)

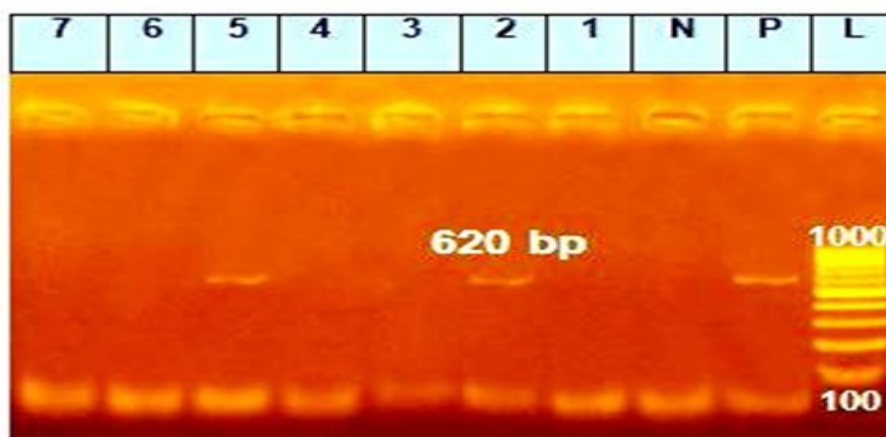


Fig 1.PCR result for the virulence gene *cnf1* on an agarose gel electrophoresis . Lanes 2and 5: *cnf1* gene positivity with 620 bp band

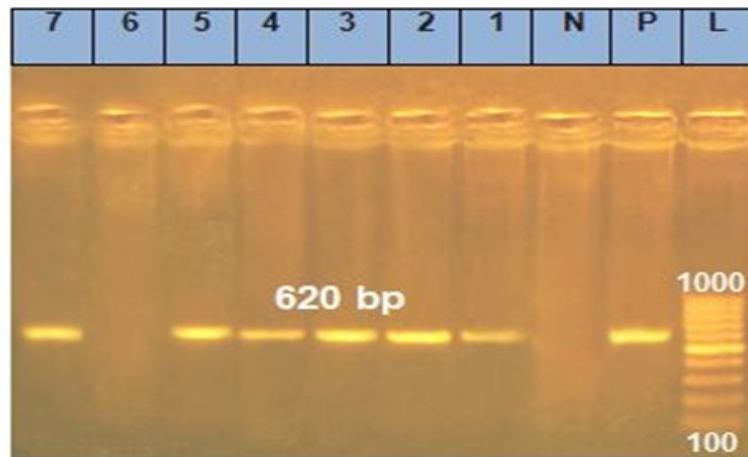


Fig 2. *tsh* virulence gene agarose gel electrophoresis of the PCR result. Lanes 1, 2, 3, 4, 5 and 7: *tsh* gene positive with 620 bp band.

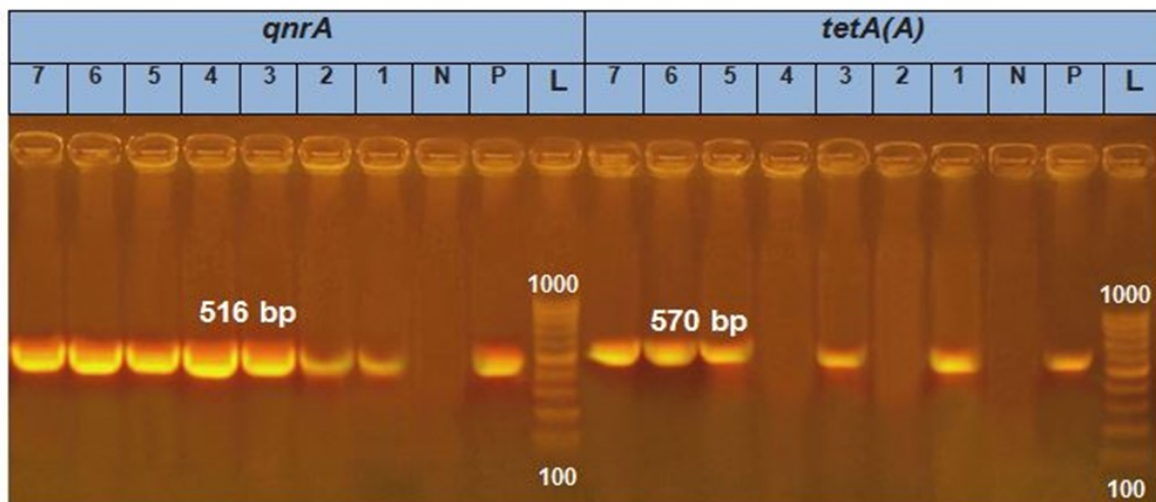


Fig 3. PCR results for the antibiotic resistance genes *qnrA* and *tetA(A)* on an agarose gel electrophoresis. Lanes 1, 2, 3, 4, 5, 6 and 7: *qnrA* gene positivity with a 516 bp band. Lanes 1, 3, 5, 6 and 7: *tetA(A)* gene positive with 570 bp band.

Experimentally infected rabbits with *E. coli* clinical signs and postmortem lesions.

Clinical signs :

The animals in all infected groups showed decrease in food intake, emaciation, apathy, fever, diarrhea or pasty feces with frequent urination. increase water intake and decrease body gain (as shown in figure 4). The severity of symptoms were reduced in group 5 (infected ,treated with acetic acid & ciprofloxacin), than group 3 (infected & treated with ciprofloxacin), than group 4 (infected & treated

with acetic acid),than in group 2 (positive control) where the symptoms were severe, the symptoms reduced gradually and disappeared nearly at 14 day post infection in all groups.

Fecal swabs

Fecal swabs were taken from four infected groups (group 2, group 3, group 4 and group 5) after one day of oral inoculation indicated fecal shedding of the *E. coli* and from diarrhea which started from day 2 of infection and stopped at day 7

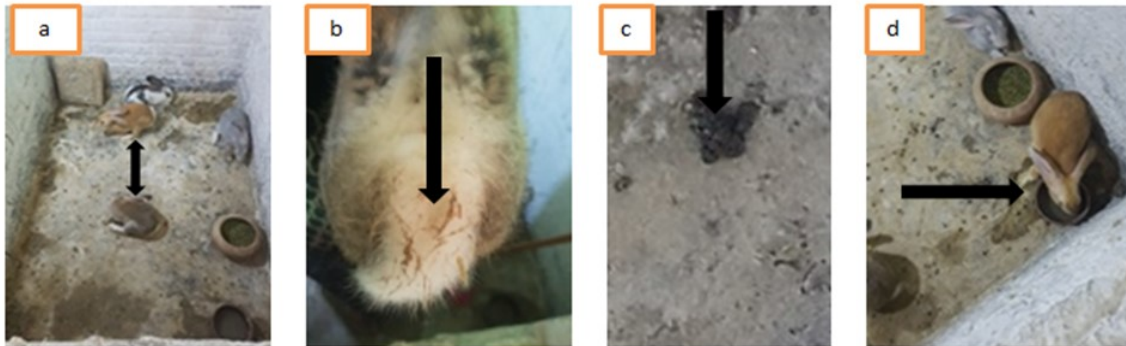


Fig (4) : Clinical signs of infected rabbits showed emaciation , apathy, shortness of breath, fever, decrease food intake and weight loss (a) , diarrhea (b) , pasty feces (c) and opaque urine, frequent urination and increase water intake (d)

Postmortem lesions :

Postmortem lesions of infected rabbits in all infected groups were taken at 7day of infection by slautaring 2 rabbits from each group& reisolation of *E.coli* from internal organs of experimentally infected rabbits were done. The infected rabbits in all infected groups showed

congested enlarged liver, enlarged gall bladder, congested heart, congested lungs, congested spleen and intestine which filled with gases and watery content (as shown in figure 5 .

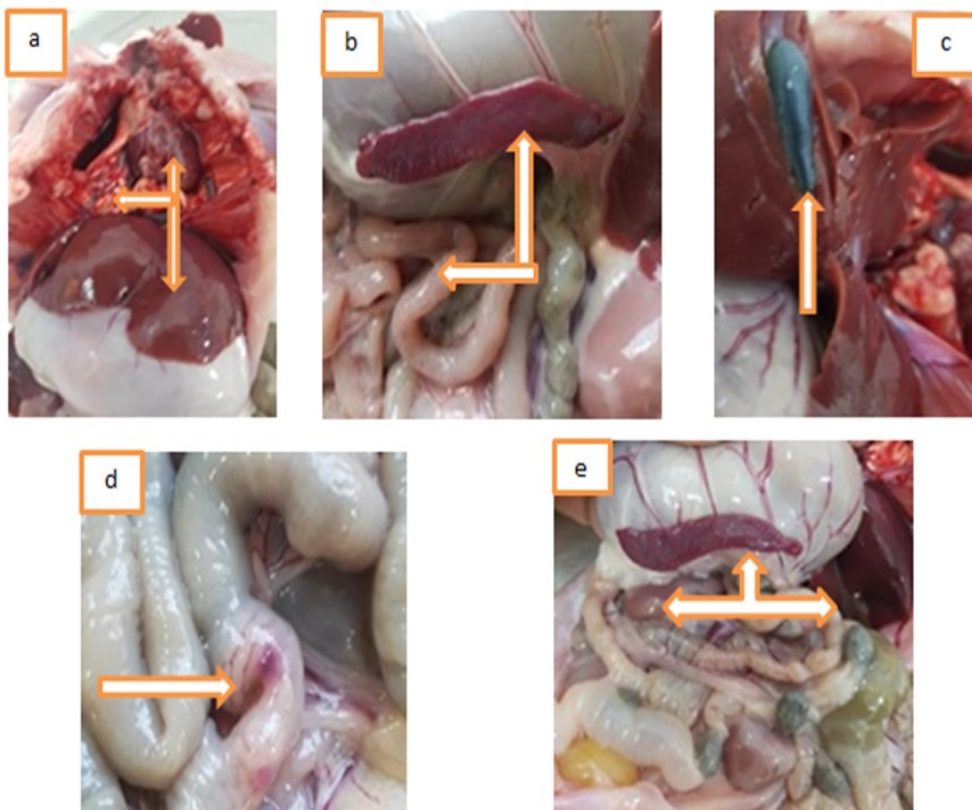


Fig (5): Postmortem lesions of infected rabbits showed : congested liver , heart & lungs (a) ,congested spleen & intestine (b) , enlarged gall bladder (c) , congested intestine (d) and congested viscera (e)

DISCUSSIONS

Incidence of *E. coli* infection in rabbit Samples

The data illustrated in table (2) clarified that the numbers of positive samples of *E. coli* from rabbit fecal swabs gathered from sick and internal organs from freshly dead rabbits in different localities at Kafrelsheikh governorate (Egypt). *E. coli* was found in 60 samples in a total percentage of 66.6%. These results resembled to **Alton et al. (2013)** who, at a rate of 61%, isolated *E. coli* from fecal samples obtained from adult 141 New Zealand white rabbits, **Sawsan, (2012)** who isolated it in a percentage of 64% from freshly dead rabbits. However, we achieved higher results than it was acquired by **Saif-Eldin et al. (1994)** who recorded *E. coli* isolation by 54% from Rabbits with diarrhea that were 4–8 weeks old had a 70–78% death rate, Also, **El-Masry & Tamam, (2021)** who isolated 54% of the *E. coli* from diarrheal rabbit fecal samples in different parts of the Egyptian governorates of Cairo, Giza, and Qalubia. Season variation, sample type, and the use of hygienic procedures on the farm could all be contributing factors to the difference in the results.

Serological identification

Rabbit diarrhea is caused by highly pathogenic strains that may be quickly identified using O serogrouping and biotyping. These strains fall into 12 different biotypes (**Pisoni et al. 2004**). Serotyping of *E. coli* isolates as displayed in table (3,5) indicated that 7 isolates of *E. coli* were serologically categorized into (three O91, two O128, one O17 and one O44 *E. coli*). Different serotypes were reported in rabbit by others as O111, O114 and O125 **Scaletsky et al. (1984)**, **Shahin et al. (2011)** discovered that the most common *E. coli* serotypes in rabbits were O44 and O158. Also O78, O125, O152, O158, O114, O115 & O168 serotypes were detected in rabbit by **Aboelhadid et al. (2022)**. The variations in *E. coli* serotypes from various sources could be the cause of the difference in the results. **Luo et al. (2023)** demonstrated that strains from several farms in the same area had different serotypes, and it's likely that other farms had the same

strains.

Antimicrobial susceptibility test

Testing for susceptibility in vitro revealed that the isolates of *E. coli* were extremely sensitive to Ciprofloxacin, Amikacin and Fosfomycin by 100%, while the strains were 90 % resistant to Tetracycline and 40% to Streptomycin table (4), analogous finding were detected by **Sakr et al. (2019)** who showed that antimicrobial sensitivity of isolated *E. coli* showed the highest sensitivity to Gentamycin 88.57% and Streptomycin 65.71% but different results were obtained by **Aboelhadid et al. (2022)** who declared that the isolates showed total resistance to neomycin (100%), and high resistance to the majority of the tested antimicrobials, considerable sensitivity to amikacin (40%) and ciprofloxacin (43.3%) was observed

Prevalence of some Virulence & antibiotic resistance genes of *E. coli* isolates

The virulence genes of *E. coli*, such as those that encode adhesion, pathogenicity islands, and outer membrane proteins, are linked to the bacteria's pathogenicity. Many *E. coli* virulence genes interact with one another during invasion to allow the bacteria to break free and destroy the host defense system, which triggers an inflammatory reaction in the host (**Ugwu et al. 2020**).

The current investigation used the pcr approach table (5), fig (1, 2, 3) to screen *E. coli* isolates for the presence of the virulence genes *cnf1* and *tsh* moreover, the antibiotic resistance genes *qnrA* and *tetA(A)*.

A protein toxin called Cytotoxic Necrotizing Factor 1 (*Cnf1*) which can induce necrosis in rabbit skin and multinucleation (cytotoxicity) in cultured cells (**Wu et al. 2007**). Our results showed that two isolates only having *cnf1* with carrying rate of 28.5% , these findings conflict with (**Sakr et al. 2019**) who discovered no *E. coli* serotype from rabbits had *cnf1* in it.

Tsh is an autotransporter of serine prote-

ase from *E. Coli* (Goudarztalejerdi et al. 2020) demonstrating that the sickness was mostly caused by the strains' ability to adhere, use transport systems, or activate enzymes. And it was noted that this gene contributed to the clones' development of multidrug resistance (Dhanji et al. 2011). Our study showed that six isolates having *tsh* with carrying rate of 85.7% this comes in agreement with a study showed that *tsh* was found in all *E.coli* isolates (Saad Eldin & Reda , 2016). A lower finding was noted by Huynh et al. (2021) who showed that *tsh* found in *E. coli* strains of rabbits by 57.14%

The *qnrA* is antibiotic resistance gene, it is the first identified plasmid-mediated quinolone resistance gene that encodes DNA gyrase protection protein so increasing prevalence will increase resistance (Wu et al. 2007). Our result clarified that the seven *E.coli* isolates carried *qnrA* gene with a carrying percent 100 The result was greater than Zhao et al. (2018) who provide that the quinolone-resistance genes, *qnrS* were presented by (5.5%) and Qing et al. (2006) who detected *qnrS* by (59.8%).

It was reported that *tetA* gene display phenotypic resistance to tetracycline antibiotic (Pereira et al. 2020) and this come in agreement with our study which showed that 5 isolates carried the gene with a carrying rate 71.4% and invitro resistance for tetracyclines by 90% .

Experimental infection :

The signs and postmortem lesions of infection appeared in all infected groups but the severity of signs and postmortem lesions were reduced in group 5 (infected , treated with acetic acid & ciprofloxacin), than group 3 (infected & treated with ciprofloxacin), than group 4 (infected & treated with acetic acid), than in group 2 (positive control) where the symptoms were sever

Clinical signs :

The animals in all infected groups showed decrease in food intake , emaciation , apathy,

fever ,diarrhea or pasty feces with frequent urination ,increase water intake and decrease body gain fig (4). These symptoms almost identical to that found by Coussement et al. (1984)

Ismail et al. (2017) , Lateef et al. (2018), SKRYPKA et al. (2020), while Mohamed et al. (2019) mentioned mild watery diarrhea and abdominal distension

Postmortem lesions :

Postmortem lesions of infected rabbits, in all infected groups, showed congested enlarged liver, enlarged gall bladder, congested heart , congested lungs, congested spleen and intestine which filled with gases and watery content fig (5). We found similar results in this investigation to that recorded by Lateef et al . (2018), they mentioned enlargement and congestions of visceral organs, while Coussement et al. (1984) mentioned that the intestinal content was watery and yelwish, Saravia et al. (2017) who showed pale liver, distended bladder .hypremic lungs with frothy material, Blanco et al. (1997) who observed ceecal edema with light brown ceecal content with some haemorrhages in ceecal mucosa

Beneficial effect of acetic acid on rabbits

In this study the use of acetic acid to minimize effect of colibacillosis in rabbits alone or with suitable antibiotic was obvious in group 5 and group 4 which may agreed with Skřivanová and Marounek, (2002) who reported the antimicrobial activity of organic acids in rabbits, Cardinali et al. (2008) who stated that in experimentally infected rabbits, organic acids lessen the harm that both Gram-positive and Gram-negative bacteria can inflict. while Hollister et al. 1990; Scapinello et al. (2001) reported no effect of organic acid were recorded. Dietary acidifiers can actually emerge as the most well-liked and successful substitute for antibiotics to be able help improve the rabbits' development and overall health because of acetic acid has the potential to act as an antibacterial because it can lower stomach pH, which reduces the survival of *E. coli* through the stomach.

CONCLUSION

The data collected demonstrated that diarrhea related to enteritis is regarded as a serious health and economic risk factor in rabbit farms. For rabbits, the weaning age is a brief, stressful time that may be linked to an overgrowth of harmful bacteria in the intestine. Stricter sanitary and preventive measures are needed in the rabbit farming business due to the high level of *E. coli* isolation in the studied rabbits. Moreover, PCR and serotyping are regarded as quick and accurate diagnostic methods for identifying *E. coli*. The isolates of *E. Coli* were 100% susceptible to Ciprofloxacin, Amikacin, and Fosfomycin. In addition, the presence of antibiotic resistant genes *qnrA* and *tetA(A)* indicated that antibiotic use in rabbit farms should to be done so carefully. When using acetic acid (1ml /litre) in drinking water in conjunction with the right antibiotics it can help in treatment of colibacillosis infections in rabbits. There is a growing inclination towards using organic acids as a viable substitute for antibiotics to enhance the health condition and growth efficiency of rabbits

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