Animal Health Research Journal Vol. 6, No. 1, March 2018 pp. 90-97

ISSN: 2356-7767

Bacteriological studies on some Gram negative bacteria causing neonatal calf diarrhea

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Received in 27/2/2018
Accepted in 29/3/2018

Abstract

Cattle breeding are facing many problems and neonatal calf diarrhea is reported as one of cattle's major diseases since it is responsible for their economic losses.

A total of 100 fecal samples collected from diarrheic calves up to 2 months old. These samples were subjected for bacteriological, serological and molecular investigations. The isolation rate of *E. coli* was 47% followed by *Pseudomonas aeruginosa* (4%) and *Salmonella Typhimurium* (1%). Sero-grouping of *E. coli* isolates revealed the presence of o_{142}, o_{11}, $o_{27}o_{157}$, o_{119}, o_{26} and o_{127} by percentage of 25%, 16.7%, 8.3%, 8.3%, 8.3%, 4.2% and 4.2%, respectively. Multidrug resistant appeared in the most tested microorganisms. Multiplex PCR was applied for detection of virulence genes *stx1* (5/10), *stx2* (2/10) and *eae* (10/10) which detected in *E. coli* and also *mexR* (4/4) antibiotic resistance gene for *Pseudomonas aeruginosa* was detected.

Keywords: *Calf diaherea, E. coli, Pseudomonas aeruginosa, Salmonella Typhimurium, Virulence and resistance genes.*

Introduction

Calves play an important role in the animal wealth either for herd replacement or as necessary source for good quality protein to fulfill the requirements of rapid increasing population **(Zaki, 2003).**

The major enteric pathogens known to cause calf scour include bacteria such as *Escherichia coli*, *Salmonella spp.*, *Clostridium perfringens* and *Pseudomonas aeruginosa* **(Aisha, 2001; Brown *et al.*, 2007; Moustafa *et al.*, 2007 and Gharieb *et al.*, 2015).**

Antibiotic resistance is increasing among many bacterial species and is rapidly becoming a world health problem. The most important serogroups of *E. coli* causing disease in animals and human are o_{157}, o_{26}, o_{103}, o_{111}, o_{145}, o_{45}, o_{91}, o_{113},o_{119}, o_{121} and o_{128} which mostly belong to shiga toxin producing *E. coli* (STEC) pathotype **(Jenkins *et al.*, 2003 and Lin *et al.*, 2011).**

Multiplex PCR includes simultaneous amplification of more than one target gene including more than one set of primers in the same reaction mixture **(Chandra *et al.*, 2013).**

Depending on genes that encoding serogroups, and virulence factors existence, multiplex PCR has been broadly used in numerous studies for differentiation of *E. coli* pathotypes **(Müller *et al.*, 2007 and Fakih *et al.*, 2016).** Also, salmonella causes diarrhea which is a complicated phenomenon, and its production of enterotoxins is one of its several pathogenic mechanisms.

Many genes like *mexR* and *blaVIM,* has been detected in many *P. aeruginosa* isolates. These genes were found to have significant percent of resistance to β-lactam antibiotics. Also, the immunological and biochemical parameters were affected as well **(Awad *et al.,* 2017).** So, the current work aimed to study the bacteriological and molecular characters of some Gram negative bacteria in diarrheic neonatal calves.

Materials and Methods

Sample collection

A total number of 100 fecal samples collected from diarrheic cattle and buffalo calves of less than 2 months old. Each sample was collected in a separate sterile container, identified and inoculated in Carry and Blair transport medium kept in an ice box and returned back to the laboratory for bacterial culturing and identification.

1- Bacteriological examination

The collected samples were cultured onto sheep blood agar and MacConkey agar. The inoculated plates were incubated aerobically for 24-48 hours (h) at 37ºC. The growing suspected colonies were picked up and tested for Gram's reaction. Colonies showed Gram negative bacilli were tested for catalase, oxidase and coagulase. The positive colonies were identified by Vitek2 compact **(Chatzigeorgiou *et al.*, 2011 and Quinn *et al.,* 2011).**

2- Serological identification

Serological identification of *E. coli* is carried out according to **Edwards and Ewing (1972),** using rapid diagnostic *E. coli* antisera set (Denka sieken comp. LTD)

The isolated *Salmonella* strain was serologically identified as *Salmonella Typhenurium* according to Kauffman-White Scheme **(Kauffman, 1974).**

3- Antimicrobial sensitivity test

The isolates were subjected to the sensitivity test against different types of antibiotics using the Vitek2 system **(Chatzigeorgiou *et al.*, 2011).**

4- Molecular examination

DNA extraction of the selected isolates (10) *E. coli* and (4) *P. aeroginosa*

DNA extraction from isolates was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations and according to **(Sambrook *et al.,* 1989).** Briefly, 200 µl of the sample suspension was added to 10 µl of the proteinase K and 200 µl of the lysis buffer and incubated at 56˚C for 10 min. Then, 200 µl of 100% ethyl alcohol was added to the lysate. After washing and centrifuging the sample, 100 µl of elution buffer that provided by the kit was used to elute the nucleic acid.

PCR amplification

PCR amplification of different ribosomal DNA of virulent genes of *E. coli* and resistance genes of *Pseudomonas aeruginosa* were carried out using the following primers (Table1). This PCR amplification was applied on 10 random isolates of *E. coli* using primers that revealed to (*stx1, stx2*, and *eae*) genes, and applied on four isolates of *Pseudomonas aeruginosa* using primer that revealed to *mexR* gene. These primers were utilized in a 25 µl reaction containing 12.5 µl of PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of nuclease-free water, and 6 µl of DNA template. The reaction was performed in an (Applied Biosystem Thermal Cycler). Cycling conditions of the different primers during the PCR amplification as the manufacturer's recommendations as follow: primary denaturation: 94˚C-5 min., secondary denaturation: 94˚C-30 sec., annealing: 55˚C-45 sec., extension: 72˚C-45 sec., no. of cycles: 35 and final extension: 72˚C-10 min.

Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the PCR products was loaded in each gel well. The fragments sizes were determined using a gelpilot 100bp and 100bp

plus DNA Ladders (Qiagen, Germany, GmbH). The gel was photographed by a gel documentation system (Alpha Innotech, Biometra).

Table (1). Primers used for the detection of virulent genes of *E. coli* and resistant genes of *Pseudomonas aeruginosa,* F: Forward and R: Reverse

Target genes	Primers sequences	Amplified Segment (bp)	Reference
stx1	F:ACACTGGATGATCTCAGTGG	614	**Shetty *et al.*, (2012)**
	R:CTGAATCCCCCTCCATTATG		
stx2	F:CCATGACAACGGACAGCAGTT	779	**Shetty *et al.*, (2012)**
	R:CCTGTCAACTGAGCAGCACTTTG		
eae	F: ATG CTT AGT GCT GGT TTA GG	248	**Bisi-Johnson *et al.*, (2011)**
	R: GCC TTC ATC ATT TCG CTT TC		
mexR	F: GCGCCATGGCCCATATTCAG	637	**Sánchez *et al.*, (2002)**
	R: GGCATTCGCCAGTAAGCGG		

Results

1- The results of bacteriological examination

E. coli was isolated as 47 out of 100 faecal samples with an infection rate of 47% followed by *Pseudomonas aeruginosa* as 4% and *Salmonella Typhimurium* as (1%).

Table (2). Prevalence of G-ve bacteria isolated from fecal samples of diarrheic calves

No. samples examined	Isolated bacterial species					
	E. coli		***P. aeruginosa***		***S. Typhimurum***	
	No.	**%**	**No.**	**%**	**No.**	**%**
100	47	47	4	4	1	1

2- The results of serotyping of *E. coli* isolated from diarrheic calves

Serogrouping of some *E. coli* isolates (24) revealed presence of o_{142}, o_{111}, o_{27}, o_{157}, o_{119}, o_{26} and o_{127} with percentage of 25%, 16.7%, 8.3%, 8.3%, 8.3%, 4.2% and 4.2%, respectively and 6 isolates were untyped as shown in table (3).

Table (3). Serotyping of *E. coli* isolated from diarrheic calves

***E. coli* serotypes**	**No. of tested strain (24)**	**% of serotypes**
o_{142}	6	25
o_{111}	4	16.7
o_{27}	2	8.3
o_{157}	2	8.3
o_{119}	2	8.3
o_{26}	1	4.2
o_{127}	1	4.2
Untyped	6	25

No.: Number of isolates and %: Percentage in relation to No. of tested isolated strains *E. coli* (24)

3- The results of antimicrobial sensitivity test. The result analysis showed antibiotic sensitivity for both *E. coli* (10) and *P. aeruginosa* (4) by Vitek2 system is shown in table (4)

Table (4): Result analysis showed antibacterial sensitivity for *Enterobacteriaceae*

Antibiotic	*E. coli* isolates(Random isolates)										*P. aeruginosa* isolates			
	1	2	3	4	5	6	7	8	9	10	1	2	3	4
ESBL	-	+	-	-	-	-	-	-	-	-	-	-	-	-
Ampicillin	S	R	S	R	R	R	R	R	S	S	S	R	R	R
Amoxicillin/ clavunic acid	S	S	S	R	S	S	S	S	S	S	R	R	R	S
Piperacillin	S	S	S	R	S	S	R	S	S	S	S	R	R	R
Cefalexin	S	S	S	R	S	R	R	R	S	S	R	R	R	R
Cefpodoxime	S	S	S	R	S	S	S	S	S	S	S	R	S	S
Ceftiofur	S	S	S	R	S	S	S	S	S	S	R	R	R	S
Cefpirome	S	S	S	R	S	S	S	S	S	S	S	S	S	R
Imipenen	S	S	S	R	S	S	S	S	S	S	S	S	R	S
Amikacin	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamicin	S	S	S	R	S	S	S	S	S	S	S	R	R	R
Tobramycin	S	S	S	R	S	S	S	S	S	S	S	R	R	S
Enrofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	R	R
Marbofloxacin	S	S	S	S	S	S	S	S	S	S	S	S	R	S
Tetracycline	S	S	S	R	S	S	R	S	S	S	S	R	R	R
Nitrofurantion	S	S	R	S	S	S	S	S	S	R	S	R	R	S
chloramphenicol	S	R	S	R	R	R	R	R	S	S	S	R	R	R
Trimethoprim/ sulfamethoxazle	S	R	S	R	R	R	R	R	S	S	R	R	R	S

R: resistant and S: sensitive.

4- The results of molecular identification

Virulence genes of *E. coli*

This PCR amplification was applied on 10 random isolates of *E. coli* for the detection of the virulence genes. Only four samples were positive to *stx1*, one sample was positive to *stx2*, one sample was positive to both *stx1* and *stx2,* and three samples were negative to both *stx1* and *stx2* (Figure 1). Meanwhile, all the ten samples were positive to the intimin gene (*eae*) of *E. coli* (Figure 2).

Resistance genes of *Pseudomonas aeruginosa*

Detection of antibiotic resistant genes of *P. aeruginosa* isolates (4) revealed that all isolates were positive for *mexR* gene as shown in Fig (3).

Table (5) illustrated the distribution of both virulence and antibiotic genes

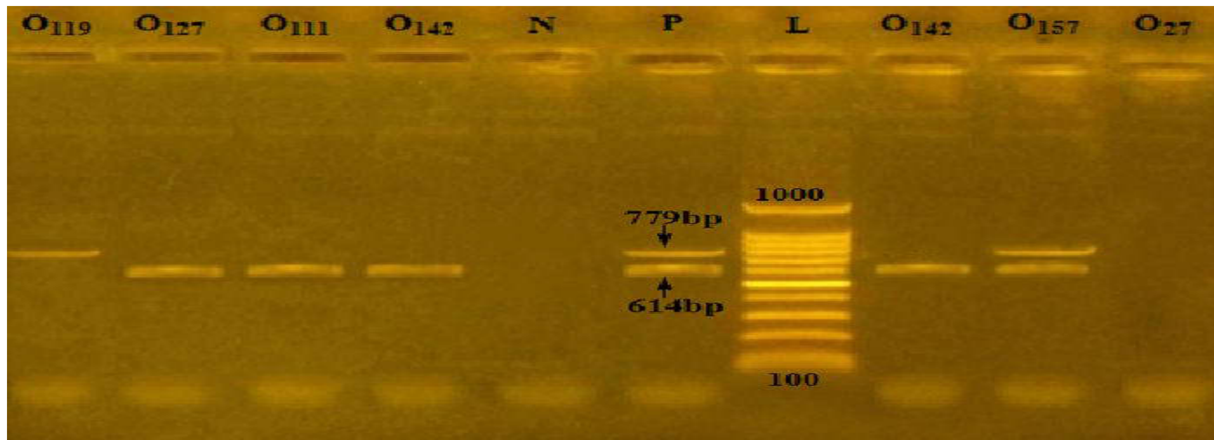

Fig. (1): Agar gel electrophoresis showed results of multiplex PCR for detection of (*stx1* which amplified at 614bp and *stx2* which amplified at 779bp) genes from samples, L: represented the molecular size marker (100pb plus ladder), N: negative control, P: positive control of these genes, o_{27}: was negative for both *stx1* and *stx2*, o_{157}: was positive for both genes, o_{119}: was positive for *stx2* and o_{127}, o_{111} and o_{142} were positive for *stx1*.

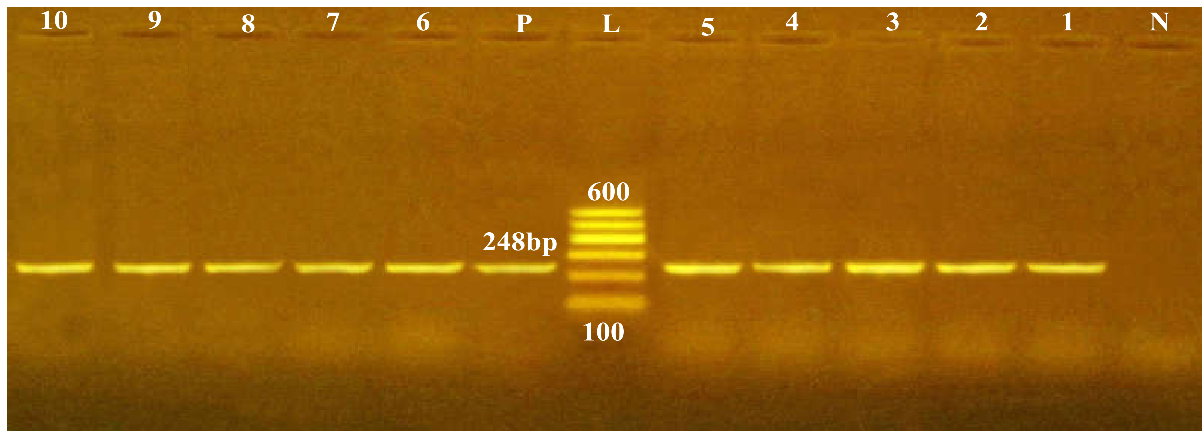

Fig. (2): Agarose gel electrophoresis showed results of multiplex PCR for detection of *eae* gene, L: represented the molecular size marker (100pb plus ladder), N: negative control, P: positive control of *eae* gene (248bp), and (Lane 1 to 10): positive to *eae.*

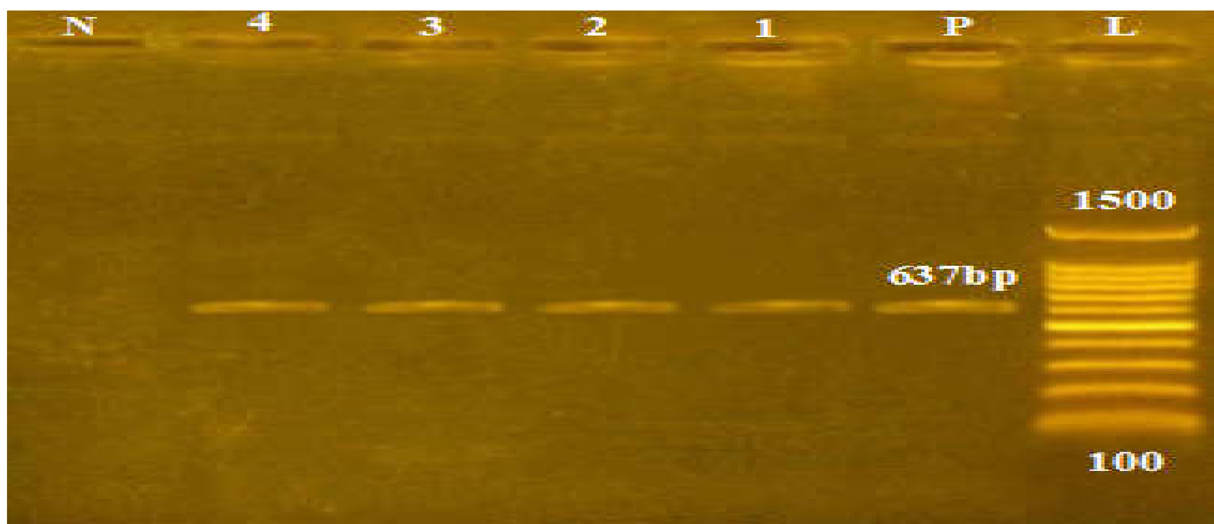

Fig. (3): Agarose gel electrophoresis showed results of multiplex PCR amplification of *P. aeruginosa* extracted DNA for *mexR* gene, L: represented the molecular size marker (100-1500bp DNA ladder). N.: negative control, P.: positive control of *mexR* gene (637bp), and Lanes: 1, 2, 3 and 4 were positive to *mexR* gene.

Table (5). The distribution of virulence and resistance genes from isolated microorganisms.

The isolated microorganisms	***E. coli*** **N=10**			***Pseudomonas aeruginosa*** **N=4**
Gene	*Stx1*	*Stx2*	*eae*	*Mex*R
Positive samples	5	2	10	4

N: number of tested isolates

Discussion

Neonatal calf diarrhea is considered as of the most important health problems in livestock causing high economic losses worldwide either directly due to mortality and needs for treatment or indirectly through poor growth **(El-Seedy *et al*., 2016).**

In this study, *E. coli* was isolated with an isolation rate of 47% as the main causative agent of family *Enterobacteriaceae* associated with diarrhea. This result was in agreement with that described by **Islam *et al.* (2015),** who isolated *E. coli* with an incidence of 45.2%.

Pseudomonas aeruginosa was isolated from the diarrheic calves in a rate of 4%, this result is in agreement with **Ashraf (2007)** who isolated it from diarrheic calves at a percentage of 4.9%.

Serogrouping of 24 *E. coli* strains out of 47 isolates revealed that 18 (75%) were belonged to eight (O) serogroups o_{142}, o_{111}, o_{27}, o_{157}, o_{119}, o_{127} and o_{26}) and 6 (25%) were serologicaly untyped strains. From eight O serogroups identified, o_{142} was the most prevalent serogroup (25%) followed by o_{111} at a rate of 16.7%. Then o_{27}, o_{157} and o_{119} at a rate of 8.3% each, and the last two serogroups o_{127} and o_{26} were found at the same rate of 4.2%.

The above mentioned results agreed with results of **Lin *et al.* (2011)** who detected o_{157}, o_{26}, o_{142} and o_{111} and, **Aisha (2001)** who isolated o_{26}, o_{127} and o_{27}.

Pseudomonas spp. isolated from diarrheic calves was *Pseudomonas aeruginosa,* this result was in harmony to that recorded previously with **Moustafa *et al.* (2007).**

In the present study *E. coli* isolates were showed two of them resistant to at least one antimicrobial agent (Table 4). Multidrug resistant was appeared on 4 strains which were similar to that obtained by **Messaï *et al.* (2013).**

In the current work, *Pseudomonas aeruginosa* multidrug resistant to most antimicrobials agreed with previously work of **Fekadu (2010)** and **Ogunleye (2012).**

Molecular characterization of *E. coli* isolated from diarrheic neonatal calves through applying different conditions of multiplex PCR for detection of genes encoding virulence factors (*stx1*, *stx2* and *eae*).

E. coli strains carried different virulence genes, as the negative isolates of *E. coli* for tested virulence genes may be non pathogenic and the animals had diarrhea caused by other infectious agents or these isolates may carry other virulence genes not included in this study. The result is nearly similar to that obtained by **Pourtaghi *et al.*, (2013).**

In this study Salmonella was isolated in low incidence of 1%, which is in agreement with **Ashraf, (2007) and Asmaa, (2015).**

In this study, multiplex PCR assays approved the presence of intimin (*eae*) 10/10 and Shiga

toxins (*stx1* 5/10 and *stx2* 2/10) in *E. coli* strains (10) which was agreed with **Gharieb *et al.*, (2015).**

The resistant gene for *P. aeruginosa* (*mexR*) was detected by PCR (4/4) which agreed with the results of **Zhao and Hu (2015), and Awad *et al*. (2017).**

Conclusion and Recommendations

1- *E. coli* and its virulence genes *stx1*, *stx2* and *eae* are responsible for diarrhea in the examined calves, and virulence genes have a major role in *E. coli* pathogenicity. On the other hand, *Pseudomonas aeruginosa* which is considered a real cause of diarrhea revealed the presence of drug resistance gene *mexR*.

2- *E. coli*, *Salmonella* and *P. aeruginosa* are major G-ve bacteria causing neonatal calf diarrhea resulting in great losses among these animals and consequently economic losses.

3- Strict hygienic measures should be applied in animal farms, intensive care for neonates and pregnant dams with their vaccinations before labor in protection of neonates from pathogenic bacteria in the first weeks of their life.

4- Neonates should be supplied colostrums just after labor which contain antibodies against pathogenic bacteria and protect these animals from infection.

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