Bacteriological and molecular studies on some bacterial agents from neonatal calf diarrhea

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A B S T R A C T

Neonatal Calf diarrhea(NCD) is a commonly reported disease and considered as a major cause of economic loss to cattle producers. This study was done on 100 fecal swabs collected from diarrheic calves(0 day-2monthes) old during the period from December 2015 till January 2017 and subjected for bacteriological ,serological and molecular investigations. The infection rate of *E.coli* was 47% followed by *Pseudomonas aeruginosa*(4%)and *Salmonella Typhimurum*(1%). Multi drug resistant appeared in the most tested microorganisms. Serogrouping of *E. coli* revealed the presence of O_{142} ,O_{55} ,O_{11},O_{27}, O_{157},O_{119} ,O_{26} and O_{127} by a percentage of 20.69%,17.24%,13.8%,6.9%,6.9%.6.9%,3.45% and 3.45%, respectively. Multiplex PCR was applied for detection of virulence genes *stx1(5/10),stx2(3/10) and eae(6/10))* which detected in *E.coli* and *stn* gene in *Salmonella typhimurum* and also ***(****blaVIM*(3/4), *mexR(4/4)***)** genes for *Pseudomonas aeruginosa* were detected

Key words: Calf diarrhea, *E.coli, Pseudomonas aeruginosa, Salmonella typhimurum, Virulence &resistance genes.*

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1. 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*, *Pseudomonas aeruginosa* (Brown *et al*., 2007),

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).

It has been widely used in various studies for differentiation of *E. coli* pathotypes based on presence of genes encoding virulence factors (Müller *et al*., 2007) and serogrouping of *E. coli* based on presence of genes encoding serogroups (Fakih *et al*., 2016)

Salmonella induced diarrhea is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxin. This enterotoxin production is mediated by the *stn* gene which is responsible for the maintenance of membrane composition and integrity(Lee *et al*.,2009)

P. aeruginosa isolates including *mex*R and *bla*VIM genes showed considerable percent of resistance to carbabenem group and other classes of β-lactam. In addition, the values of biochemical and immunological parameters were affected (Awad *et al*., 2017) .So the current work aimed to study the bacteriological & molecular characters of some aerobic bacteria in diarrheic neonatal calves.

2. Materials and methods

2.1 Sample collection:

A total number of 100 fecal swabs from diarrheic cattle and buffalo calves (30 samples from buffalo calves and 70 from cattle calves) of less than 2 months old at different seasons (winter and summer) during the period from December 2015 till January 2017 were examined bacteriologically.

The samples were collected, labeled and transported as soon as possible in ice box to the laboratory for bacteriological examination.

*2.2 Bacteriologic*al *and biochemical examination*:

Swabs were inoculated in Carry and Blair transport media and returned back to the laboratory for bacterial culturing and identification The collected samples were cultured onto sheep blood agar and Macconkey agar. The inoculated plates were incubated for 24-48 hours at 37^{o} C. The suspected colonies were picked up and tested for Gram's reaction. Colonies showed Gram negative bacilli were tested for catalase and coagulase. The positive colonies were tested by VITEK2 compact (Quinn et al.,2011)

2.3. Antimicrobial sensitivity test:

The isolates were subjected to the sensitivity test against different types of antibiotics, using the Vitek 2 system (Chatzigeorgiou *et al.*, 2011)

2.4 Serological identification

Serological identification of *E.coli* is carried out according to Edwards and Ewing (1972) "table 3".

2.5. Molecular examination:

PCR amplification of different ribosomal DNA of virulent genes of both *E.coli* and salmonella and resistant genes of *Pseudomonas aeruginosa* were carried out using the following primers (table1)

By using QIAamp® DNA Mini Kit instructions (Catalogue no. M501DP100) (Sambrook *et al*, 1989). It was applied on 10 random isolated *E.coli (stx1 , stx2, eae)* gene PCR ,and applied on one *salmonella typhimurum* isolate (*stn*)gene PCR also applied on four isolate of *Pseudomonas aeruginosa* (*bla*VIM-*mex*R)

3. RESULTS

3.1. The results of bacteriological examination

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

3.2. The results of Antimicrobial sensitivity test:

The results of Antimicrobial *sensitivity test* showed distribution of multi drugs resistant bacteria in most tested strains (table2)

3.3. The results of serotyping of E.coli isolated from diarrheic calves

Serogrouping of *E. coli* isolates (29) revealed presence of O_{142} ,O_{55} ,O_{11}, O_{27}, O_{157}, O_{119} ,O_{26} and O_{127} by a percentage of 20.69%,17.24%,13.8%,6.9%,6.9%.6.9%,3.45 % and 3.45% respectively

3.4. The results of molecular identification

3.4.1. Virulence genes

3.4.1.1. Virulence genes of E.coli

Four samples were positive to *stx1*&two samples were positive to *stx2* and one sample was positive to both *stx1* and *stx2* and two samples were negative to both *stx1&stx2* (Figure 1& 2) of 10 random examined samples of *E.coli* meanwhile O142,O26,O119,O55 and O157 were positive to the intimin gene (*eae)* but O27,O111&O127 were negative to *eae* (*eae*) of *E.coli*(Figure3)

3.4.1.2. The results of *Salmonella typhinurum* virulence genes using PCR

enterotoxin(*stn*) gene was detected in Salmonella *typhinurum* which is virulent gene responsible for pathogenicity (Figure4)

3.4.1.3.The results of *Pseudomonas aeurignosa* resistance genes identifications.

Four samples were positive to *mex*R and three samples were positive to *bla* VIM as showen in (Figure 5).

The distribution of both virulence and resistance genes were showed in table(4)

Table (1):primers used for the detection of virulent genes of *E.coli* and s*almonella* and resistant genes of *pseudomonas aeruginosa* F:Forward (3`-5`),R: Reverse(5`-3`)

get ıe	Primers sequences	Amplified Segment (bp)	Reference
	F:ACACTGGATGATCTCAGTG G	614	Dipineto *et al.*, 2006
	R:CTGAATCCCCCTCCATTATG		
	F:CCATGACAACGGACAGCAGTT	779	
	R:ACACTGGATGATCTCAGTGG		
	F: ATG CTT AGT GCT GGT TTA GG	248	Bisi-Johnson *et al*., 2011
	R: GCC TTC ATC ATT TCG CTT TC		
IM	F: TTTGGTCGCATATCGCAACG	500	Amudhan *et al.*, 2012
	R:CCATTCAGCCAGATCGGCAT		
:R	F: GCGCCATGGCCCATATTCAG	637	Sánchez *et al*., 2002
	R:ATT CGT AAC CCG CTC TCG TCC	617	Murugkar *et al*., 2003
	F:TTG TGT CGC TAT CAC TGG CAA CC		

Table (2): Distribution of multidrug resistant bacteria

Resistance patterns	*E. coli* n=13		*Pseudomonas aeruginosa* n=4		*Salmonella typhimurum* n=1	
	No	%	N0	%	No	%
To one drug	2	15.3	-	-	-	-
To only two drugs	-	-	-	-	-	-
To only three drugs	2	15.3	1	25	-	-
To more than three drugs	6	46.15	3	75	1	100
To all drugs	-		-	-	-	-

No.: Number of isolates. %: Percentage in relation to No of tested isolates

N= number of samples

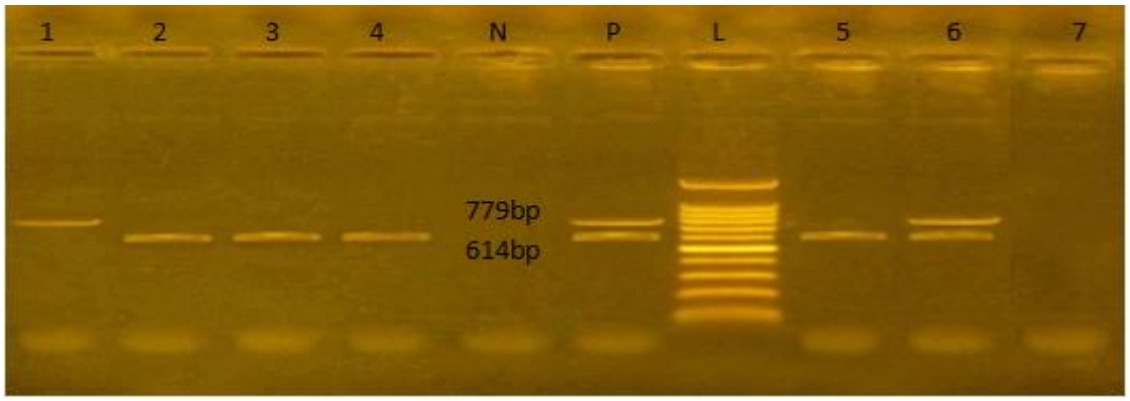
Table (3): Serotyping of *E.coli* isolated from diarrheic calves

E.coli serotypes	Number (out of 13)	% of serotypes
O142	6	20.69
O55	5	17.24
O111	4	13.8
O27	2	6.9
O157	2	6.9
O119	2	6.9
O127	1	3.45
O26	1	3.45

No.: Number of isolates. %: Percentage in relation to No of tested isolated strain (*E.coli* (29)

Table (4) Distribution of virulence &resistance genes from isolated microorganisms.

The isolated M.O	*E.coli* n=10			*Salmonella typhimurum* n=1	*Pseudomonas aeruginosa* n=4	
Gene	*Stx1*	*Stx2*	*eae*	*stn*	*Mex* R	*Bla* VIM
Positive samples	5	3	6	1	4	3

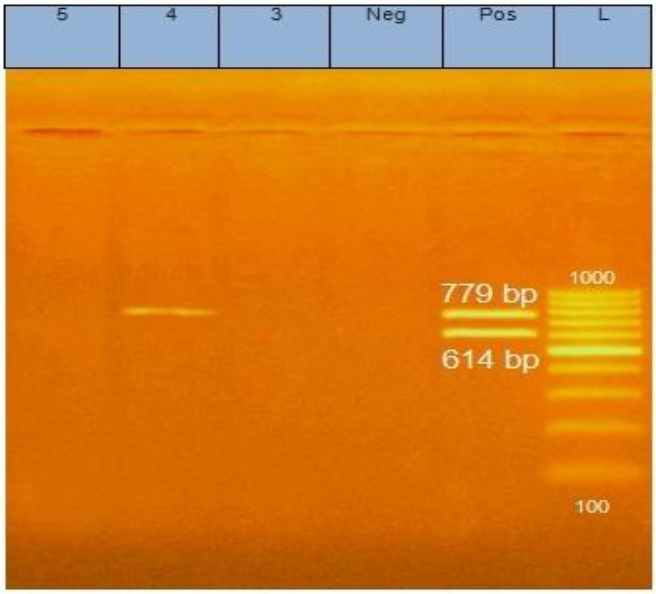


Fig(1): Agar gel electrophoresis showed results of multiplex PCR for detection of (*stx1* which amplified at 614 bp and *stx2* which amplified at 779 bp) genes from samples No(1-7) L: represent the molecular size marker(100pb plus ladder)

Lane 1 positive to *stx2* (O119) Lane 2,3,4,5 positive to *stx1*(O127-O111-O124-O124)

Lane 6 positive to *stx1*&stx2(O157) -Lane 7 negative tostx1&*stx2*

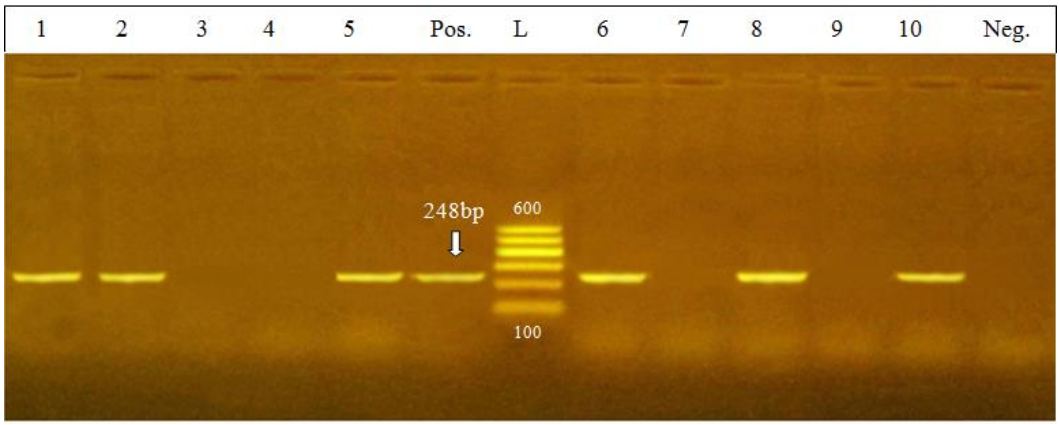
N : control negative P:control positive



(Fig 2)Agar gel electrophoresis showed results of multiplex PCR for detection of stx1 and stx2 genes from *E. coli* samples No.(3 to 5)

L: represent the molecular size marker (100pb plus ladder) 3: negative for stx1 & stx2 (O55)

4: positive of stx2 779 bp (O55) 5- negative for stx1 & stx2 (O26)



(fig3)Agarose gel electrophoresis showed results of uniplex PCR for detection of *eae* gene

Neg : Negative control. Pos: Positive control of *eae* gene(248 bp)

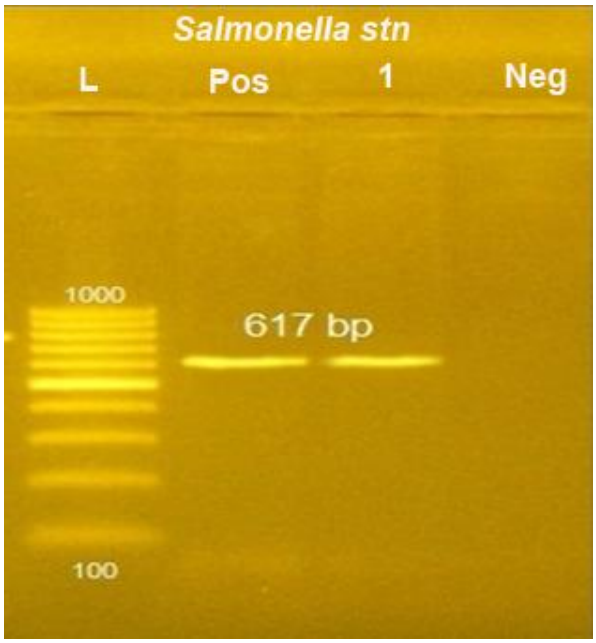
L: represents the molecular size marker (100pb plus ladder)

(Lane1,2): Positive for o142 Lane 6 Positive for O119

(Lane 3.7) Negative for: O27&O127 Lane 8: Positive for O55

(Lane 4) negative for ,O111 Lane 9: Negative for O55

(Lane 5) Positive for,O26 L ane10, Positive for O157



Figure(4)Agarose gel electrophoresis of uniplex PCR amplification *Salmonella typhinurum* of extracted DNA.

L: represents the molecular size marker (100-1000bp DNA ladder).

Neg.: Negative control Pos.: Positive control of stn (617bp)

1; positive for stn gene (617bp)

(a) mexR (b) BlaVIM

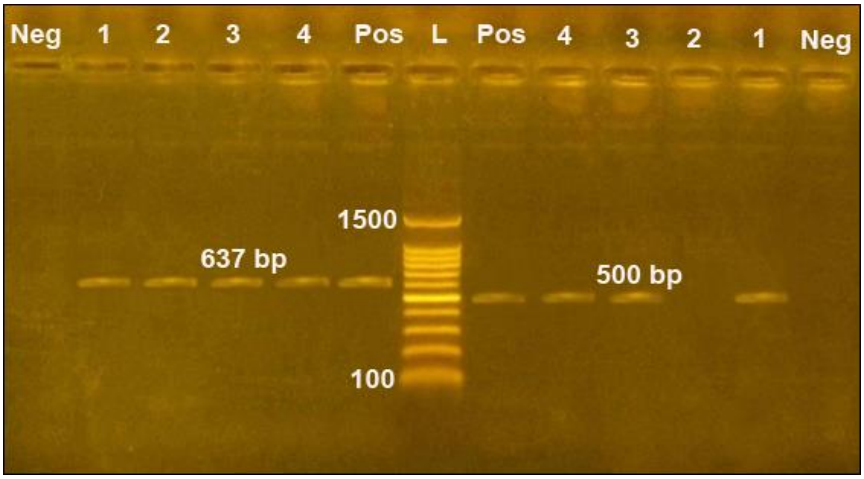


Figure (5): Agarose gel electrophoresis of multiplex PCR amplification of *Ps. aeruginosa* extracted DNA

(a): (*mex R*) gene. (b): (*blaVIM*) gene.

L: represents the molecular size marker (100-1500bp DNA ladder). Neg.: Negative control

Pos(b).: Positive control of *bla*VIM (500bp)

Pos(a).: Positive control of *mex*R (at 637bp)

Lane (a): 1a ,2a ,3a ,4a positive for *mexR* gene (637bp)

Lane (b): 1b, 3b , 4b positive for *bla*VIM gene(500bp)

Lane (2a) negative for *bla*VIM gene(500bp)

4. DISCUSSION

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

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

The *Pseudomonas aeruginosa* which isolated from diarrheic calves in percentage of 4%. This result was similar to the result obtained by Ashraf(2007) who isolated *Pseudomonas aeruginosa* from calves with diarrhea at apercentage of 4.9% .

Although Salmonella spp is considered an important causative agent of NCD. In the present study it was isolated with low infection rate(1%). The obtained result was nearly agreed with that recorded by Ok *et al*,(2009) and Asmaa,(2015) with an incidence of 1.2% and 0.8%, respectively.

The *Salmonella* spp isolated from calves with diarrhea serotyped as *Salmonella typhimurium*. This result agreed with Nabih and Arafa,(2012).

Serogrouping of 47 *E.coli* isolates using genes of 29 strains O serogroups O_{142},O_{55},O_{111},O_{27},O_{157},O_{119},O_{127} and O_{26} revealed that 79.3% of *E.coli* strains were belonged to eight O serogroups and 20.7% were belonged to un identifiable serogroup (nontypable). From eight O serogroups identified, O_{142} was the most prevalent serogroup 20.7%) followed by O_{55} and O_{111} at rate of 17.24% and 13.8% respectively ,then O_{27},O_{157} and O_{119} at a rate of 6.9% and the last two serogroups $O_{127}$and O_{26}were found at the same rate 3.45%.

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 were *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 (2). Multidrug resistant was appeared on eight strains were similar to that obtained by Messaï *et al.(* 2013).

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

Multidrug resistance present in the isolated sample of *Salmonella typhimurum* was agreed with results of Yhiler and Bassey(2015).

Molecular characterization of *E.coli* isolated from diarrhea in neonatal calves through applying different conditions of uniplex and 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, the rate of *stx* gene in isolated *E.coli* strains from cattle and buffalo calves was 30%. Multiplex PCR assays approved the presence of intimin $(eae)_{6/10}$ and Shiga toxins ($STx1_{5/10}$ *and* $STx2_{3/10}$) in *E.coli* strains$_{(10)}$ which was agreed with Gharieb *et al.* (2015). In the current study *E.coli* O_{157} was positive to *stx1, stx2* and eae genes, this agree with Karmali,(2004).

Salmonella induced diarrhea is a complex phenomenon involving several pathogenic mechanisms including production of enterotoxins.

This enterotoxin production is mediated by the *stn* gene which is responsible for the maintenance of membrane composition and integrity (Chopra *et al*,1994).

The *stn* gene is present in *Salmonella typhimurum(1/1)* and this result is agreed with that observed by Moore and Feist,(2007) and Lee *et al*. (2009).

The resistant genes for *P. aeruginosa* (*Mex*R and *bla*VIM) were detected by PCR agreed with results of Zhao and Hu,(2015)and Awad *et al*,(2017).

5. CONCLUSION

The main cause of diarrhea in the examined calves was *E.coli* and its virulence genes was *stx1,stx2* and *eae* which played an important role in its pathogenicity while,

Salmonella which considered an important causative agent of NCD was recorded at low percentage with virulence gene (*stn*) ,On the other hand, *Pseudomonas aeruginosa* which is considered a real cause of diarrhea revealed presence of drug resistance genes *(bla* VIM-*mex* R) against beta-lactimase.

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