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-2 months) 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 Typhimurium* (1%). Multi drug resistant appeared in the most tested microorganisms. Serogrouping of *E. coli* revealed the presence of O142, O55, O11, O27, O157, O119, O26 and O127 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 typhimurium* and also (blaVIM (3/4), mexR(4/4)) genes for *Pseudomonas aeruginosa* were detected

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

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 O137, O26, O103, O111, O145, O45, O91, O113, O119, O121 and O128 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 mexR and blaVIM genes showed considerable percent of resistance to carbapenem 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 Bacteriological 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°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 (table 1)

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 typhimurium isolate (stn)gene PCR also applied on four isolate of Pseudomonas aeruginosa (blaVIM-mexR).

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 (44%) and Salmonella typhimurium 1 (1%).
3.2. The results of Antimicrobial sensitivity test:

The results of Antimicrobial sensitivity test showed distribution of multidrug resistant bacteria in most tested strains (table 2).

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

Serogrouping of E. coli isolates (29) revealed presence of O142, O55, O11, O27, O157, O119, O26 and O127 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 (Figure 3).

3.4.1.2. The results of Salmonella typhimurium virulence genes using PCR

Enteroxotoxin (stn) gene was detected in Salmonella typhimurium which is virulent gene responsible for pathogenicity (Figure 4).

3.4.1.3. The results of Pseudomonas aeruginosa resistance genes identifications.

Four samples were positive to mexR and three samples were positive to bla VIM as shown 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 salmonella and resistant genes of pseudomonas aeruginosa  F:Forward (3’-5’), R: Reverse(5’-3’)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Amplified Segment (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IM</td>
<td>F:TTTGGTCGCATATCGCAACG</td>
<td>500</td>
<td>Amudhan et al., 2012</td>
</tr>
<tr>
<td></td>
<td>R:CCATTGCAGCCAGATCGGCAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>:R</td>
<td>F:GCGCCATGGCCCATATTCCAG</td>
<td>637</td>
<td>Sánchez et al., 2002</td>
</tr>
<tr>
<td></td>
<td>R:ATT CGT AAC CCG CTC TCG TCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F:TTG TGT CGC TAT CAC TGG CAA CC</td>
<td>617</td>
<td>Murugkar et al., 2003</td>
</tr>
<tr>
<td></td>
<td>F:ACACTGGATGATCTCAGTG</td>
<td>614</td>
<td>Dipineto et al., 2006</td>
</tr>
<tr>
<td></td>
<td>R:CTGAATCCCCCCTCATTATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F:CCATGACAACGGACAGCAGT</td>
<td>779</td>
<td>Bisi-Johnson et al., 2011</td>
</tr>
<tr>
<td></td>
<td>R:ACACTGGATGCTCAGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F:ATG CTT AGT GCT GGT TTA GG</td>
<td>248</td>
<td></td>
</tr>
</tbody>
</table>
Table (2): Distribution of multidrug resistant bacteria

<table>
<thead>
<tr>
<th>Resistance patterns</th>
<th>E. coli (n=13)</th>
<th>Pseudomonas aeruginosa (n=4)</th>
<th>Salmonella typhimurum (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>No</td>
</tr>
<tr>
<td>To one drug</td>
<td>2</td>
<td>15.3</td>
<td>-</td>
</tr>
<tr>
<td>To only two drugs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>To only three drugs</td>
<td>2</td>
<td>15.3</td>
<td>1</td>
</tr>
<tr>
<td>To more than three drugs</td>
<td>6</td>
<td>46.15</td>
<td>3</td>
</tr>
<tr>
<td>To all drugs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>E. coli serotypes</th>
<th>Number (out of 13)</th>
<th>% of serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>O142</td>
<td>6</td>
<td>20.69</td>
</tr>
<tr>
<td>O55</td>
<td>5</td>
<td>17.24</td>
</tr>
<tr>
<td>O111</td>
<td>4</td>
<td>13.8</td>
</tr>
<tr>
<td>O27</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>O157</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>O119</td>
<td>2</td>
<td>6.9</td>
</tr>
<tr>
<td>O127</td>
<td>1</td>
<td>3.45</td>
</tr>
<tr>
<td>O26</td>
<td>1</td>
<td>3.45</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>The isolated M.O</th>
<th>E. coli (n=10)</th>
<th>Salmonella typhimurum (n=1)</th>
<th>Pseudomonas aeruginosa (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Stx1</td>
<td>Stx2</td>
<td>eae</td>
</tr>
<tr>
<td>Positive samples</td>
<td>5</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>
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 to stx1&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 (100 pb plus ladder)

(Lane 1, 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  Lane 10: Positive for O157

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

L: represents the molecular size marker (100-1000 bp DNA ladder).
Neg.: Negative control  Pos.: Positive control of stn (617 bp)
1: Positive for stn gene (617 bp)
4. DISCUSSION

Neonatal calf diarrhea is considered as one 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 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 a percentage 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 O142, O55, O111, O27, O157, O119, O127 and O26 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, O142 was the most prevalent serogroup 20.7% followed by O55 and O111 at rate of 17.24% and 13.8% respectively, then O27, O157 and O119 at a rate of 6.9% and the last two serogroups O127 and O26 were found at the same rate 3.45%.

The above mentioned results agreed with results of Lin et al. (2011) who detected O157, O26, O142 and O111 and Aisha (2001) who isolated O26, O127 and O27.

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 (STx1510 and STx2310) in E. coli strains10 which was agreed with Gharieb et al. (2015). In the current study E. coli O157 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 (MexR and blaVIM) 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 (\textit{sti}) . On the other hand, \textit{Pseudomonas aeruginosa} which is considered a real cause of diarrhea revealed presence of drug resistance genes (\textit{bla VIM-mex R}) against beta-lactimase.

6. REFERENCES


Murugkar, H.V.; Rahman, H. and Dutta, P.K. (2003): Distribution of virulence


