Molecular exploring of multidrug resistance update of E. coli causing neonatal calves’ diarrhea.

Aya M. Esmail¹, Amal M. Rgab², Amira M. Rizk¹

¹Bacteriology, Immunology and Mycology Department, Faculty of Veterinary Medicine, Benha University, Egypt.
²Animal Health Research Institute, Tanta branch, Egypt.

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ABSTRACT

Cattle producers usually face considerable economic losses due to neonatal calf diarrhea (NCD). Aiming to delineate this problem, we conducted bacteriological, serological, and molecular studies on 100 fecal swabs collected from diarrheic calves (1 day to 3 months) old from different farms in Gharbia Governorate, Egypt, from December 2021 to May 2022. The E. coli isolation rate was 34%, with the multi-drug resistance being a general trend in most isolates. Sero-grouping of E. coli resulted in the encountering of O1, O26, O44, O55, O115, O125, O127, O128 (5% for each type), O18, O119 (10% for each type) and K99 (25%). Virulence and resistance genes molecular investigation using PCR in 2 isolates of E. coli serotype(K99) revealed the presence of virulence genes (LT, STα) and resistance genes (qnrA, tuc(6)-IB-cr, blaTEM, KPC, SUL, and aadA). We concluded that the higher the stress the E. coli face from improperly using anti-bacterial drugs, the more advanced and new mechanisms the bacteria develop to overcome this challenge. Therefore, multi-drug resistant strains of E. coli are continuously emerging, posing serious threats to human and animal health.

1. INTRODUCTION

Diarrhea in calves is a common devastating clinical sign of pre-weaning infection by gut microbial agents. This is caused by multiple pathogens such as Escherichia coli, Clostridium perfringens, Salmonella enterica species, Rotavirus, Bovine coronavirus, Cryptosporidium species and Eimeria species. Some factors may predispose to the development of such disease, such as failure to acquire passive immune protection from mothers through colostrum and other intertwined environmental factors. Of the most important factors that contribute to the incidence of calf scour are the different determinants that affect intestinal permeability, especially in the first hours and days after birth, as calves with higher diarrhea incidence were reported to have higher intestinal permeability in the first 2 hours after birth compared to calves that do not have scours (Araujo et al., 2015). This was suggested due to variations in the development of calves’ GIT microbiome from different maternal fluids and tissues during the parturition process (Taschuk and Griebel, 2012). In addition to the host genome, which affects the population of the commensal bacteria, it impacts the GIT microbiota establishment microbiota (Benson et al., 2010).

Newborn calf death due to diarrhea in the first few days of the calves’ life reached 57%, as reported by the National Animal Health Monitoring System (NAHMS) for U.S. dairy report in 2007 (Cho and Yoon, 2014). This devastating death rate, in addition to the high costs paid for the diagnosis, treatment, labor, and veterinary intervention, imposes a high economic loss on the whole economic profit of the dairy or fatting business. In Egypt, bovine newborn E. coli infection is the primary cause of calf death, which ranges between 27.4% and 55% of the mortality in newborn calves (Ahmed et al., 2009). The ability of any pathogenic agent to produce gut disturbance depends on its ability to colonize the GIT and destroy the host tissue.

To produce diarrhea, E. coli rely on its adhesion antigens that facilitate the bacteria to hang on enterocytes. The enterotoxigenic E. coli (ETEC) was accused of being the primary cause of calves’ diarrhea in the 1st four days of life due to oral exposure to fecal matter contaminated by coliforms. This results in GIT colonization with commensal flora that continues to move caudally with the ingesta. Ingesta with a high ETEC load with the virulence factors K99 fimbria, and heat-stable toxin will produce diarrhea. The fimbrial antigen K99 enables the ETEC to attach to and colonize the intestinal wall (Foster and Smith 2009). This attachment to the intestinal wall allows the bacteria to maintain lodging and expansion to the extent that about 80% of the organisms are attached to the intestinal wall in the calves having scour compared to only about 20% being attached in the calves without scour (Hadad and Gyles 1982). After colonizing the ileum, ETEC proliferates and attains the ability to secret a heat-stable toxin that causes secretory diarrhea (Sherwood et al., 1983; Constable, 2003). This heat-stable toxin (STα) binds to an intestinal vill brush.
From December 2021 to May 2022, fecal swabs were taken from 100 diarrheal calves (aged one day to 3 months) from various farms in the Gharbia governorate. Without waiting, all swabs were transmitted through transport media and transported in an ice box to the lab for bacteriological analysis.

2.2. Bacteriological and biochemical examination:

Each fecal swab was inoculated in 3 mL of peptone broth (Oxoid, UK) and incubated for 24 h at 37 °C according to Quinn et al. (2002), a loopful from broth was inoculated into MacConkey’s agar (Oxid, UK) and incubated at 37 °C for 18–24 h. Lactose fermenter (pink) colonies were streaked onto eosin methylene blue agar (EMB) (Oxid, UK) and identified by cultural and morphological characteristics, then validated as E. coli using the standard biochemical tests according to Collee et al. (1996).

2.3. Serological identification:

By performing a slide agglutination test with conventional polyvalent and monovalent E. coli antisera (Denka Seiken- Co., Ltd., Tokyo, Japan), E. coli isolates were identified serologically according to Edward and Ewing (1972).

2.4. Antimicrobial sensitivity test: E. coli isolates were tested for their sensitivity to 8 different antimicrobial discs (Oxoid, UK), including amoxicillin-clavulanic acid (20+10 μg), linezolid (30 μg), gentamycin (10 μg), ceftiraxone (30 μg), meropenem (10 μg), trimethoprim-sulfamethoxazole (1.25+23.75 μg), norfloxacin (10 μg) and levofloxacin(5μg) by using disc diffusion method on Mueller Hinton agar according to Clinical and Laboratory Standards Institute (CLSI, 2021).

2.5. Molecular examination:

Two selected E. coli isolates (K99) were examined for detection of virulence genes (LT, StA) and resistance genes (qnrA, aac(6)-IB-cr, blaTEM, KPC, sul, adaB). By using QIAamp® DNA Mini Kit instructions (Catalogue no.51304). The PCR program used the primers shown in Table (1) and running conditions as detailed in Table (2). PCR products were separated by electrophoresis in 1% agarose gel. Bands were visualized by ethidium bromide (Sambrook et al. 1989). The run was stopped after about 30 min, and the gel was transferred to the UV cabinet. A gel documentation system photographed the gel, and the data was analyzed through computer software.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5’ - 3’)</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>qnrA</td>
<td>F- ATTTTTGACGTCGAGGATTG&lt;br&gt;R- GATCGGCAAAGTAGTTCACA</td>
<td>516 bp</td>
<td>Shams et al., 2015</td>
</tr>
<tr>
<td>aac(6)-IB-cr</td>
<td>F- TGGGATGGTCTATGAGTGGCTA&lt;br&gt;R- CTCAGATGGCGCGGTTT</td>
<td>482 bp</td>
<td>Hu et al., 2013</td>
</tr>
<tr>
<td>blsTEM</td>
<td>F- ATGGGATGTCACATGCGGT&lt;br&gt;R- TTACCAATGTTAACAGTGAGG</td>
<td>861 bp</td>
<td>Azam et al., 2016</td>
</tr>
<tr>
<td>KPC</td>
<td>F- ATGCTTGTCGCTCTAGT&lt;br&gt;R- GCCGCGGCTCTCG</td>
<td>796 bp</td>
<td>Yigit et al., 2001</td>
</tr>
<tr>
<td>Sul</td>
<td>F- TGGCCGAAGCTCTG&lt;br&gt;R- TTTTTCGCGGCG</td>
<td>433 bp</td>
<td>Tosini et al., 1998</td>
</tr>
<tr>
<td>adaB</td>
<td>F- GAGCGAAATCCTG&lt;br&gt;R- TGGCGATGCTCTATGGC</td>
<td>404 bp</td>
<td>Frana et al., 2001</td>
</tr>
<tr>
<td>LT</td>
<td>F- ATGGGATGTCACATCGCTT&lt;br&gt;R- ATTTTGATGTCG</td>
<td>132 bp</td>
<td>Nishikawa et al., 2002</td>
</tr>
<tr>
<td>StA</td>
<td>F- ATTTTGTTCGCTATCT&lt;br&gt;R- CACCGGTCAGGAGT</td>
<td>190 bp</td>
<td>Stacy et al., 1995</td>
</tr>
</tbody>
</table>
3. RESULTS

3.1. Bacteriological examination
Out of 100 fecal swabs, 34 E. coli isolates were detected and identified via morphological characters (Gram negative non sporulated motile bacilli), cultural characters (lactose fermenter pink colonies on MacConkey’s agar and green metallic sheen colonies on EMB agar) and biochemically identification (positive result with Indole and Methyl red tests while Voges Proskauer, Citrate, Urease and H2S tests were negative).

3.2. Serotyping of E. coli isolates
Serotyping of 34 E. coli isolates showed that 01, O26, O44, O55, O115, O125, O127, and O128 were present in two isolates (5.88 %) for each type, 3 isolates (8.82%) for both O18, O119, and 8 isolates K99 (23.53%), and 4 isolates untypable (11.76%).

3.3. Antimicrobial sensitivity test
All E. coli (34) isolates show (100%) resistance to amoxicillin-clavulanic acid (20+10 μg), ceftriaxone (30 μg); linezolid (30 μg) and trimethoprim-sulphamethoxazol (1.25+23.75 μg). On the other hand, the sensitivity to meropenem was (100%), 35.3 % (12/34) to gentamycin (10 μg), 20.6% (7/34) to levoflaxacin (5μg),and 5.9 % (2/34) to norfloxacin (10 μg).

3.4. Molecular identification
Detection of virulence genes: The tested two E. coli K99 isolates (No. 1, 2) were positive to the virulence genes (LT gene bands at 132bp, and STa gene bands at 190bp) (figure 1, 2 respectively).

3.5. Detection of resistance genes of E. coli K 99:
The E. coli K99 isolates No. (1 & 2) testing for resistance genes (qnrA, aac(6)-IB-cr, blaTEM, Sul and aadB) revealed the expression of all these genes in both isolates at 516, 482, 861,433 and 404 bp. respectively (figure 3, 4, 5, 7, 8). While KPC gene expression was detected in isolate K99 No.1 only (gene bands at 798 bp) and absent in isolate No. 2. (figure 6).
Fig (7): Agarose gel electrophoresis showing PCR amplification of sul gene bands at 433 bp. L: marker (100bp plus ladder), N: +ve control, P: -ve control. *Lane (1-2): No. of
E. coli (K99) isolates (were positive for sul gene)

Fig (8): Agarose gel electrophoresis showing PCR amplification of aadB gene bands at 404 bp. L: marker (100bp plus ladder), N: +ve control, P: -ve control. *Lane (1-2): No. of
E. coli (K99) isolates (were positive for aadB gene)

* Positive and negative controls were represented by field sample that were previously confirmed to be positive or negative by PCR for the related genes in the Reference Laboratory. Animal Health Research Institute, Egypt.

4. DISCUSSION

ue to calves’ diarrhea, bovine breeders suffer significant financial losses, which poses a major threat to the cattle industry (Cho and Yoon 2014). In this research, deciphering the bacterial causes of the newborn calf scours in the studied area revealed E. coli is the leading cause as its isolation rate was 34%, consistent with the previous isolation rate of E. coli of 37% from calf diarrheic samples in Ethiopia (Gebregiorgis and Tessaema 2016) and a 28.8% in Egypt (Algammal et al., 2020). This current study results are also parallel with a previously reported E. coli being the most predominant bacterial pathogen causing newborn calves’ diarrhea (Cho and Yoon 2014). However, E. coli isolation rate in this work is far lower than the isolation rate from calves’ scour in other studies undertaken in other localities from Egypt reported an 80%, 70.7%, and 75.6% isolation rate of E. coli (El-Seedy et al., 2016; Aref et al., 2018; Awadet et al., 2020). However, the E. coli isolation rate in the current work is far higher than other reported isolation rates in other countries, as 13.5% was reported in a study in Ethiopia (Darsema 2008) and 5.5% in Switzerland (Uhde et al., 2008). This discrepancy between the rates of E. coli isolation from different localities and countries may be attributed to the differences in management and control programs and environmental conditions. It also indicates the uncontrolled and hazardous use of antibiotics in food animal management and prophylaxis programs in Egypt. It stresses the bacteria, pushing it to develop drug resistance to overcome routine management and control measures and guarantee its presence and multiplication. It also should represent an alarming problem that necessitates nationwide strictly implemented rules to reduce antibiotics’ uncontrolled and hazardous use.

The serotyping results of the presence of O1, O26, O44, O55, O115, O127, and O128 (5% for each type), 10% for both O18, O119, and K99 (25%) representing 85% with the presence of 15% un-serotyped isolates is consistent with a previous study (Algammal et al., 2020). This study’s recognized serotypes differ from previously reported serogroups isolated and sampled from diarrheic newborn humans and animals (Uhde et al., 2008; Vaghand Jani, 2010; Enany et al., 2019). Moreover, the pattern and distribution of serotypes retrieved in the study are nearly similar to the recently reported serogroups of isolates from Egypt (Algammal et al., 2020).

Heat-labile (LT) and heat-stable (STa and STb) toxins are the mediators by which Enterotoxigenic E. coli (ETEC) induce its pathogenicity in humans and animals (Olaogun et al., 2016). Investigation of E. coli isolates in this study showed their expression of these two toxin-encoding genes, proving its biotyping as ETEC type and indicating its pathogenicity. ETEC toxin LT activates adenylate-cyclase enzyme in the affected animal’s enterocytes, while STa acylivates guanylate-cyclase enzyme, leading to severe watery diarrhea. The encounter of these two toxins-encoding genes in all isolates comes in accordance with a previous study that showed the expression of (LT) in all tested E. coli isolates either alone or in association with the STa, pointing to their importance in ETEC-pathogenicity (Algammal et al., 2020).

The improper and hazardous use of antibiotic drugs for prophylaxis and infection treatment in food animals and poultry has led to the emergence of the globally high threat spread of antimicrobial-resistant (AMR) bacterial strains (Ahavasi et al., 2023). E. coli can attain resistance genes by horizontal gene transfer (HGT) through interspecies and intra-species routes (Poirel et al. 2018). The E. coli’s HGT mechanisms include plasmids, transposons, and pathogenicity islands (PAIs) insertion into the recipient E. coli genome (Javadi et al. 2017).

Complicating this harmful scenario, E. coli can work not only as a recipient of HGT from other bacteria but also as a donor of resistance genes to other bacteria (Poirel et al., 2018), thus acting as an essential player in the widespread drug-resistance genes among pathogenic bacteria. Compared to a recent study on diarrheic E. coli from Egypt (Algammal et al., 2020), the current study showed a higher multi-drug resistance pattern as 100% of the E. coli isolates (34) were resistant to amoxicillin-clavulanic acid (20+10 μg), ceftriaxone (30 μg); linezolid (30 μg) and trimethoprim-sulfamethoxazole (1.25+23.75 μg), 65% to gentamycin (10 μg), 80% to levofloxacin (5μg) and 95% to norfloxacin (10 μg) compared to 50% resistance to trimethoprim/sulfamethoxazole (50%) and (42.8%) resistance to gentamycin (Mousa et al., 2010). This can be attributed to increasing microbial drug resistance development or differences in antibiotic stress on E. coli due to differences in the management and preventive control measures in other studied localities. Recent studies have reported strains of E. coli with advanced resistance to most antibiotic classes (Xiao et al. 2019, Zhang et al. 2021). As E. coli is considered the most active and smart bacteria for developing drug-resistance genes, the more advanced the antibacterial stress it faces, the more resistant genes emerge. The problem is expected to be seriously complicated when these new genes are transmitted to other pathogenic bacteria, representing a health concern in animals and humans. An example of this scenario is the multi-drug resistance of the isolated bacteria in the current study, as all antibacterial drugs were either totally or partially ineffective whenever the only drug that could efficiently inhibit E. coli growth and multiplication was meropenem. This agrees with a previously reported 100% sensitivity of E. coli isolates to meropenem (Wu et al., 2021). Moreover, the 100% resistance of the currently studied isolates (34) to amoxicillin/clavulanic acid is far higher than the recently reported 21.5% resistant E. coli (Algammal et al., 2020). This multi-drug resistance ability
of E. coli isolates is mediated through the presence of drug resistance genes (qnrA, aac (6)-Ib-cr, blaTEM, sul, and aadB) that were shown in this study to be expressed in all the examined isolates. The discrepancy between the drug-resistance pattern in this work and the previously reported one may be caused by the presence of blaTEM, sul, and aadB resistance genes at a higher rate in this work compared to a lower expression rate in the previous study (Algammal et al., 2020). This work consisted of the expression of multi-drug-resistance genes aadB, sulI, and blaTEM genes in all the tested isolates; these genes were reported to be prevalent in all the tested isolates resistant to aminoglycosides, sulfonamides, and ampicillin (Du et al., 2005).

5. CONCLUSIONS

This study throws some light on the importance of continuously updating the knowledge of the constantly inventing and developmental changes of the microbial community in each locality. Moreover, E. coli remains the main cause of calf diarrhea, resulting in substantial financial losses. The higher the stress the E. coli are facing by improperly using anti-bacterial drugs, the more advanced and new mechanisms the bacteria develop to overcome this challenge. Therefore, multi-drug resistant strains of E. coli are continuously emerging which pose severe threats to human and animal health. The differences between different localities in the pattern of drug resistance are related to the levels of improper antibiotic use in food animals and poultry prophylaxis and management in such areas.

6. REFERENCES


