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Development of PCR and Multiplex-PCR for Detection of Some *Escherichia coli* Virulence Genes from Bovine Neonates Diarrhea

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ABSTRACT

Colibacillosis is a fetal and serious problematic disease particularly in young calves during the first days of their life accompanied with high mortality losses. The aim of this study is to moleculary characterize *Escherichia coli (E. coli)* strains isolated from individual cases of neonatal calf diarrhea. Characteristic clinical symptoms as yellowish diarrhea with low colostrum feeding history was observed during samples collection. Rectal swabs were obtained from 50 healthy and 100 clinically infected diarrheic calves which identified by traditional bacteriological and serological procedures. Then, *E. coli* isolates were subjected for genetic characterization of virulence genes (*stx1*, *stx2*, *fimH*, *iutA*, *eaeA* and *tsh*) using both uniplex and multiplex PCR. The most dominant virulence gene was *fimH* in all the examined isolates followed by *iutA* in 50% however, the *eaeA* and *tsh* genes were in 37.5% while no detection for both *stx1* and *stx2* genes. In conclusion, these results provide updated information regarding the molecular detection of *E. coli* strains from neonatal calves in Egypt and thus, would be important to formulate preventing tools and effective therapy against *E. coli* either in Egypt and or worldwide.

Keywords: *E. coli, PCR, Cattle, Diarrhea.*

INTRODUCTION

Neonatal calf diarrhea is an important etiology for higher morbidity and mortality worldwide in bovine neonates (Constable, 2004). This multifactorial disease comprised several factors as the calf immune status, environmental and farm management practices (housing, feeding and hygienic conditions) (Lorenz, 2006). *Escherichia coli* is the main causative agent which play an important role in the disease occurrence (Tenaillon *et al.*, 2010). However, a reduced number of highly adapted pathogenic *E. coli* strains are capable of causing intestinal or extra intestinal diseases associated with high economic losses (Sousa 2006). Pathogenic *E. coli* strains have different virulence factors that permit them to colonize on small intestine as well as escaping the immune system and stimulating the deleterious inflammatory response causing diarrhea (Croxen and Finlay 2010). Only the most successful combinations of virulence factors have been persisted to become specific *E. coli* pathotypes which are capable of causing disease in healthy individuals (Kaper *et al.*, 2004). These virulence factors include Shiga toxin1 (encoded by the *Stx1* gene), Shiga toxin 2 (encoded by the *Stx2* gene), intimin (encoded by the *eaeA* gene), bundle forming pilus (encoded by bfp gene), and enterohaemolysin (encoded by the Ehly gene) (Kang etal., 2004). Moreover, previous studies identified several *E. coli* virulence genes such as the *tsh* gene which encodes a temperature-sensitive hemagglutinin and the ferric aerobactin receptor (*IutA*) in spite of, expression of fimbrial (pilli) antigens was the more prominent virulence factor identified for Enterotoxigenic *E. coli* strains that help in bacteria adhesion and colonization in the luminal surface of the small bowel and responsible for elaboration of one or more enterotoxins that influence intestinal secretion of fluids (Welch 2006). The aim of the present study is molecular characterization of pathogenic *E. coli* strains isolated froma random sampling of neonatal calves suffering from dirrhea.

MATERIAL AND METHODS

Samples collection: A total of 150 faecal samples were collected from 1-30 days old neonatal calves (100 samples from individual cases of diarrhea in calves and 50 samples from apparently healthy calves) .

Bacteriological isolation and identification: Fecal samples were inoculated onto MacConkey broth then on Eosin Methylene Blue (EMB) agar medium. Colonies showing characteristic metallic sheen on EMB agar were picked up and considered as presumptive *E. coli*. The purified cultures of *E. coli* were stored temporarily as semisolid agar medium for further identification by biochemical tests, *E. coli* isolates were preliminarily characterized by IMViC tests, viz., indole, methyl red, Voges-Proskauer, and citrate utilization according to (Cowan, 1985 and Cheesbrough, 1984). The isolates which exhibited the IMViC pattern of +, +, −, and −, respectively, were presumed as *E. coli* isolates.

Serotyping:

All *E. coli* isolates were serotyped by slide agglutination test according to (Edwards and Ewings, 1972).

Molecular identification of some *E. coli* virulence genes:

DNA extraction from the samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH, Catalogue no.**51304**) with slight modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer.

PCR condition**s** and predicted sizes of the amplified fragments for the specific oligonucleotide primers used in the study were shown in (***Table 1)***. Analysis of the PCR products was carried out by agar gel electrophoresis at 50 Volt for 60 minutes using 1% agarose gel stained with ethidium bromide and visualized under UV transluminator.

Table (1). Oligonucleotide primers used for detection of *E. coli* virulence genes

Target gene	Primer sequences	Amplified fragment (bp)	Reference
eaeA	ATG CTT AGT GCT GGT TTA GG GCC TTC ATC ATT TCG CTT TC	248	Bisi-Johnson *et al.*, 2011
Stx1	ACACTGGATGATCTCAGTGG CTGAATCCCCCTCCATTATG	614	
Stx2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	Dipineto *et al.*, 2006
fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	Ghanbarpourand Salehi, 2010
Tsh	GGT GGT GCA CTG GAG TGG AGT CCA GCG TGA TAG TGG	620	Delicato *et al.*, 2003
iutA	GGCTGGACATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300	Yaguchi *et al.*, 2007

Table (2). PCR Cycling conditions for the different primers.

Target gene	Initial denaturation	Denaturation	Annealing	Extension	No. of cycles	Final extension
stx1 **and** ***stx2***	94˚C 5 min.	94˚C 30 sec.	58˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
fimH	94˚C 5 min.	94˚C 30 sec.	50˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
iutA	94˚C 5 min.	94˚C 30 sec.	63˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.
Tsh	94˚C 5 min.	94˚C 30 sec.	54˚C 45 sec.	72˚C 45 sec.	35	72˚C 10 min.
eaeA	94˚C 5 min.	94˚C 30 sec.	51˚C 30 sec.	72˚C 30 sec.	35	72˚C 7 min.

RESULTS:

Prevalence of *E. coli* isolates from young calves.

The prevalence of *E. coli* isolates from 100 diarrheic calves was (57%), while the percentage from apparent healthy calves was (52%). (Table 3)

Serological identification for *E. coli* isolates from young calves showing diarrhea

Using Serotyping, the most prevelant detected *E. coli* serogroups were O125 with (28.57%) followed by O126 (14.28%), while O146, O44, O18 and O6 in addition to rough and untyping strains (28.57%) as shown in ***(Table 4).***

Molecular characterization of virulence genes of*E. coli* strains from diarrhea cases in young calves.

In this study 8 isolates of *E. coli* were screened via uniplex and multiplex PCR for detection of *Stx1*, *Stx2*, *fimH*, *iutA*, *eaeA* and *tsh* genes. (***Table 5)*** showed that *fimH* gene was detected in all the examined isolates by percentage of 100%, 50% for *iutA* gene and 37.5% for both *eaeA* and *tsh* genes while *Stx1*, *Stx2* were not detected in any isolate.

Table (3). Prevalence of *E. coli* isolates from cattle diarrhea.

Types of animals		Positive cases for *E. coli*	
		Number	Percentage (%)
ApparentlyHealthy cases	50	26	17.33
Diarrhea cases	100	57	38
Total number	150	83	55.33

% were estimated in relation to the total collected samples (150).

Table (4). Percentage of *E. coli* serotypes in the samples under study.

Serotype	Percentage (%)
O146	7.14
O126	14.28
O125	28.57
O44	7.14
O18	7.14
O6	7.14
Untypeable	28.57

Table (5). Virulence genes of isolated *E. coli* strains of examined diarrheic samples:

Serogroups of *E. coli* strains	Animal health status	Virulence genes					
		Stx1	*Stx2*	*fimH*	*iutA*	*eaeA*	*Tsh*
O146	Diseased	-	-	+	+	-	+
O126	Diseased	-	-	+	+	+	-
O125	Diseased	-	-	+	+	-	+
Untypeable	Diseased	-	-	+	-	+	-
O44	Diseased	-	-	+	-	+	-
O18	Apparently healthy	-	-	+	+	-	+
O125	Diseased	-	-	+	-	-	-
O126	Diseased	-	-	+	-	-	-

DISCUSSION:

Colibacillosis in young calves constitutes one of major economic losses in several countries. In the present study 150 faecal samples were collected, out of 150 samples 83 samples were positive for *E. coli* (57 diseased and 26 apparently healthy) with overall prevalence rate of 55.33%. Our reults are lower than that obtained by Pourtaghi *et al.,* (2013) who collected 180 rectal swabs from neonatal diarrhoeic calves, 156 *E. coli* isolates were identified with percentage of 86.6% and Achá *et al.,* (2004) with a percentage of 76 % while are higher than that obtained by Gebregiorgis and Tessema (2016) who showed that 74 out of the 201 diarrheic calves that showed calf diarrhea were *E. coli* positive (36.8 %). The wide variation from one report to other is due to variation in geographic locations of sampling as well as different methodology used for bacterial identification such as; source and type of samples, enrichment procedure and choice of selective media (Islam *et al.*, 2014). Furthermore, farm management conditions including inadequate nutrition, exposure to severe environment, insufficient attention to the newborn calf, or a combination of these, qualitative and quantitative conditions of the colostrum, are often involved as a suggestive cause of variation (Radostits et al. 2007).

Serological identification of *E. coli* is considered a sensitive method for detection of pathogenic serotypes. In this study the most serovars identified were O125, O126, O146, O44, O18 and O6 in addition to rough and untyping strains. This also supported by other studies of Gharieb *et al.,* (2015). In the present study molecular detection of *E. coli* virulence genes; *Stx1*, *Stx2*, *fimH*, *iutA*, *eaeA* and *tsh* genes was performed through PCR. Our results revealed that *fimH* gene was detected in all examined isolates with a percentage of 100% followed by 50% for *iutA* gene, then 37.5% for *eaeA* and *tsh* genes while *Stx1*, *Stx2* were not detected in any isolate. The fmbriae gene acts as a virulence factor that enhance the adhesion and colonization of pathogenic stains of *E. coli* in mucosal surface of calves small intestine*fimH*. However, several studies demonstrated that type 1 fmbriae have been implicated for the colonization of *E. coli* in the urinary tract infection in human (Connell *et al*., 1996; Donnenberg& Welch, 1996; Mulvey *et al*., 1998). Although, other reports indicated that several fimbriae genes were also involved in *E. coli* pathogenicity as F4 (K88), F5 (K99), F6 (987P), F18, and F41 (DebRoy and Maddox, 2001).

The importance of Shiga toxins in *E. coli* pathogenicity depends on its responsibility for attachment and binding of bacteria to glycolipid on the Gb3 sites on the cell surface leading to cessation of protein synthesis and consequently death of bacterial cell (Kaper *et al.,* 2004). In addition to the main role in production of enterotoxins that exert diarrhea as well as alteration the acid-basebalance of blood and small intestine (Nagy and Fekete, 1999; , Nataro and Kaper, 1998) These results was parallel to Karmali *et al.,* (2003) who reported that *Stx1* gene was not detected, while the *Stx2* gene was found to be 93.1%. while other study examined 25 *E. coli* isolates, only 7 isolates were found positive for *Stx1* and none of them were *Stx2* positive (Akter *et al.,* 2016),however, these results were different than results obtained by Wieler *et al.,* (2007 and Abotalp *et al.,* (2017) who reported that sxt2 gene percentage range between 4.5% and 43.75% in Germany and Egypt, respectively. Awad-Masalmeh, (2004) showed that the incidence of *Stx1* and *Stx2* were 10.1% and 17.8%, respectively. The function of Intimin that encoded by *eaeA* gene in pathogenic *E. coli* isolates and other fimbriae genes can be concluded on its ability to adhere, attachment and colonize in the locus (A/E) sites of target host cell (Mainil, 2013). The present study detected *eaeA*in (37.5%). Other previous studies reported that incidence of *eaeA* gene range from *eaeA* 1.2% to 9.8% (Yuluo *et al.,* 2010; Nguen *et al.,* 2011 and Salehi *et al.,* 2011). The wide difference may be attributed due to the number of collected samples or number of examined isolates, methodology as well as sources of examined isolates.

The *tsh* gene encodes a temperature-sensitive hemagglutinin of *E. coli*. In this study, *tsh* gene was detected in 37.5% out of the screened isolates. These results are nearly in agreement with Delicato *et al.,* (2003) who mentioned that 39.5% of *E. coli* harbored *tsh* gene. In Egypt, several studies reported that the *tsh* was 28% and 100%, respectively (Mohamed *et al.,* 2014 and Abdulgayeid *et al.,* 2015). Furthermore, another study, reported that the incidence rate of *tsh* gene in *E. coli* strains obtained from avian source were 85.3%

and 78.3%, respectively (Saidenberg *et al.*, 2013). This wide range in frequency of the gene required further investigation about the actual distribution and prevalence of this gene in different animal species and its role in interspecies transmission and another related factor.

REFERENCES:

Acha, S.J.; Kuhn, I.;Jonsson, P.;Mbazima, G.;Katouli, M. and Mollby, R. (2004): Studies on calf diarrhea in Mozambique: prevalence of bacterial pathogens. Acta Vet. Scand. 45: 27–36.

Akter, M.M.;Majumder, S.;Nazir, K.H.M.N.H. and Rahman, M. (2016): Prevalence and molecular detection of shiga toxin producing *Escherichia coli* from diarrheic cattle J. Bangladesh Agril. Univ. 14(1): 63–68, 2016.

Awad-Masalmeh, M. (2004): Virulence genes of verotoxin producing non-157 *E. coli* strains isolated from healthy small ruminants and cattle (in German). Wiener TierarztlicheMonatsschrift, 91, 47–55.

Bisi-Johnson, M.A.; Obi, C.L.; Vasaikar, S.D.; Baba, K.A. and Hattori, T. (2011): Molecular basis of virulence in clinical isolates of *Escherichia coli* and Salmonella species from a tertiary hospital in the Eastern Cape, South Africa. Gut Pathogens 2011, 3:9.

Cheesbrough M (1984). Medical Laboratory Manual for Tropical Countries. vol 11, Microbiology, pp. 400- 480.

Constable, P. (2004): Antimicrobial use in the treatment of calf diarrhea.J Vet Intern Med. 2004;18:8---17.11

Connell, I.; Agace, W., Klemm, P.; Schembri, M,; Mărild, S.; and Svanborg, C.; 1996. Type 1 fimbrial expression enhances *Escherichia coli* virulence for the urinary tract.ProcNatlAcadSci U S A. Sep 3; 93(18): 9827–9832.

Cowan ST (1985). Biochemical behavior of *E. coli*. Journal of General Microbiology 8: 391.

Croxen, M. and Finlay, B. (2010): Molecular mechanisms of *Escherichia coli*pathogenicity. Nat Rev Microbiol. 2010;8:26---38.

Delicato, E.R.; de Brito, B.G.; Gaziri, L.C.J. and Vidotto, M.C. (2003): Virulence-associated genes in *Escherichia coli* isolates from poultry with colibacillosis. Veterinary Microbiology 94 (2003) 97–103.

Dipineto, L.; Santaniello, A.; Fontanella, M.; Lagos, K.; Fioretti, A. and Menna, L.F. (2006): Presence of Shiga toxin-producing *Escherichia coli* O157:H7 in living layer hens. Letters in Applied Microbiology 43 (2006) 293–295.

Donnenberg, M.S., and R.A.Welch.1996.Virulence determinants of uropathogenic *E. coli*. In:H.L.A.J.W.W.Mobley (Eds.) Urinary Tract Infections: MolecularPathogenesis and Clinical Management. ASM Press.Washington, DC.135–174.

Edwards, P.R. and Ewing, W.H. (1972): identification of Enterobacteriacae.Minneapolis, l.pp.709.Burgess publishing cp.Atlanta USA 3rd. Ed

Gebregiorgis, A. and Tessema, S.A. (2016): Characterization of *Escherichia coli* isolated from calf diarrhea in and around Kombolcha, South Wollo, Amhara Region, Ethiopia Trop Anim Health Prod (2016) 48:273–281.

Ghanbarpour and Salehi (2010): Determination of Adhesin Encoding Genes in *Escherichia coli* Isolates from Omphalitis of Chicks. American Journal of Animal and Veterinary Sciences 5 (2): 91-96, 2010.

Gharieb, M.R.; Fawzi, M.E.; Attia, E.N. and Bayoumi, H.Y. (2015): Calf diarrhea in Sharkiaprovince, Egypt: diagnosis; prevalence, virulene profilesand zoonotic potintialof the causalbacterial agents Int. J. Agric.Sc&Vet.Med. 2015.

Islam, M.Z.; Musekiwa, A.; Islam, K.; Ahmed, S.; Chowdhury, S.; Ahad, A.; et al. (2014) Regional Variation in the Prevalence of *E. coli* O157 in Cattle: A Meta-Analysis and Meta-Regression.

Kang, S. J.; Ryu, S. J.; Chae, J. S.; Eo, S. K.; Woo, G. J. and Lee, J. H. (2004): Occurrence and characteristics of enterohemorrhagic *Escherichia coli* O157 in calves associated with diarrhoea. Veterinary Microbiology 98: 323–328.

Kaper, J.;Nataro, J. and Mobley, H. (2004): Pathogenic *Escherichia coli*. NatRevMicrobiol. 2004;2:123---40.

Karmali, M.A.;Mascarenhas, M.; Shen, S.H.;Ziebell, K.; Johnson, S. *et al.,* (2003): Association of genomic O (-)island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. J ClinMicrobiol, 41: 4930-4940.

Lorenz, I. (2006): Diarrhoea of the young calf: an update. Proceedingsof the XXIV World Buiatrics Congress. 2006. p. 130---8.

Mohamed, M. A.; Shehata, M. A. andRafeek, E. (2014): Virulence Genes Content and Antimicrobial Resistance in *Escherichia coli* from Broiler Chickens. Vet. Med. Int.

Mulvey M.A.; Lopez-Boado,Y.S.; Wilson,C.L.; Roth,R.; Parks,W.C.; Heuser,J. and Hultgren,S.J. (1998) Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli.*Science, 282, 1494–1497.

Nagy, B. and Fekete, P.Z. 1999. Enterotoxigenic *Escherichia coli* (ETEC) in farm animals.Vet Res. 1999 Mar-Jun;30(2-3):259-84.

Nataro, J.P. and Kaper, J.B. 1998. Diarrheagenic *Escherichia coli*. ClinMicrobiol Rev. 1998 Jan;11(1):142-201.

Nguyen, T.D.;Vo, T.T. and Vu-Khac, H. (2010): Virulence factors in *Escherichia coli* isolated from calves with diarrhea in Vietnam. Journal of Veterinary Science 12: 159-164.

Pourtaghi, H.; Dahpahlavan, V. and Momtaz, H. (2013): Virulence genes in *Escherichia coli* isolated from calves with diarrhoea in Iran Comp ClinPathol (2013) 22:513–515.

Radostits, O. M.; Gay, C. C.;Hinchcliff, K. W. and Constable, P. D. (Eds.) (2007), Veterinary Medicine, 10th Edition, pp. 847- 888, Saunders, Philadelphia.

Salehi, Z.T.;Badouei, A.; Mahdi;Brujeni, N.; Gholamreza;Madadgar and Omid (2011): Occurrence and characterization of enterohaemorrhagic isolates *Escherichia coli* from diarrhoeic calves, department of microbiology, faculty of veterinary medicine, University of Tehran, Tehran, Iran.

Sousa, C.P. (2006): *Escherichia coli* as a specialized bacterial pathogen.RevBiolCiên Terra. 2006;6:341---52.

Tenaillon, O.;Skurnik, D.; Picard, B. andDenamur, E. (2010): The populationgenetics of commensal *Escherichia coli*. Nat Rev Microbiol.2010;8:207--17.

Welch, R.A. (2006): The Genus Escherichia, Prokaryotes, 6, 60–71

Wieler, L.H.;Sobjinski, G.;Schlapp, T.; Failing, K.; Weiss, R.;Menge, C. andBaljer, G. (2007): Longitudinal prevalence study of diarrheagenic *Escherichia coli* in dairy calves. Brel Munch TierarztlWochenscher 120:296–306

Yaguchi, K.; Ogitani, T.; Osawa, R.; Kawano, M.; Kokumai, N.; Kaneshige, T.; Noro,T.; Masubuchi, K. and Shimizu, Y.(2007): Virulence Factors of Avian Pathogenic *Escherichia coli* Strains Isolated from Chickens with Colisepticemia in Japan. Avian Dis. 2007 Sep;51(3):656-62.

Yuluo, W.U.;Hinenoya, A.; Taguchi, T.;Nagita, A.;Shima, K.; Tsukamoto, T.; Sugimoto, N.;Asakura, M. and Yamasaki, S. (2010): Distribution of Virulence Genes Related to Adhesins and Toxins in Shiga Toxin-Producing *Escherichia coli* Strains Isolated from Healthy Cattle and Diarrheal Patients in Japan. The Journal of Veterinary Medical Science 72: 589–597.