Investigation of Bacterial Species Causing Diarrhea in Calves

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Abstract

Calf diarrhoea is a multifactorial disease entity that can have severe financial animal welfare implications in both dairy and beef herds. The involvement of bacterial pathogens is the main cause of bloody diarrhoea in calves and causes high mortality and morbidity. This study aimed to isolate the bacteria causing diarrhoea and biochemical identification of the isolated bacteria. Faecal samples obtained from diarrheic calves were tested in this study, including buffalo calves and cattle calves aged from seven days to one year. The incidence varied amongst farms, ranging from 0% to 27.9%. The buffalo calves were most affected than cattle calves with diarrhoea. The calves of age from seven days to three months were the most affected calves with diarrhoea. The most isolated bacteria were *E.coli* followed by *C.pefringens* and *Salmonella* spp. were the last isolated in this study in bacteriological testing through morphological characters and biochemical identification.

Keywords : Diarrhea ,Calves ,*E.coli* ,*C.perfringens* ,*Salmonella*

Introduction

The most common cause of calf morbidity and mortality in pre-weaned calves is diarrhoea. Calves under 30 days of age have a diarrhoea rate of between 10% and 20%. ***(Bendali et al., 1999a; Svensson et al., 2003).*** Diarrhoea is a complex syndrome caused by various agents through proliferation in the intestine of newborn animals during the first few days of life as the immune system is not well developed, and the maternal immunity doesn't withstand variable infections ***(Holland, 1990).*** The involvement of bacterial pathogens is still responsible for more than 50% of cases of neonatal calf diarrhoea, and *E.coli* is more or less consistently isolated during a cultural examination of the intestinal content of calves during the first three weeks of age ***(Malik et al, 2012).*** *Salmonella* spp., *E.coli* k99, and *Clostridium* species have all been identified as bacterial agents in calves less than two months ***(Acha et al. 2004 ; Smith, 2009).*** Viruses, bacteria, and protozoa have also been implicated ***(Bhat et al .,2012;Bhat et al .,2015, single et al., 2013).*** *Escherichia coli* is a gram-negative rod-shaped motile non sporulated, flagellated and facultatively anaerobic member of

the family Enterobacteriaceae. It usually is found in the lower intestine of most warm-blooded animals. ***(Reid et al., 2001).*** *Salmonella* species are gram-negative rod-shaped facultative anaerobic bacteria of the family Enterobacteriaceae. *Salmonella* is a large genus with about 3000 different serovars ***(Davies, 2008).*** Clinical signs of systemic infections linked with diarrhoea and septicemia may be associated with *Salmonella* spp. conditions lead to mortality in extreme situations ***(Berge et al, 2008).*** Human and animal *Salmonella* infections can cause a wide range of diarrhoea, including acute gastroenteritis, bacterial infections in the bloodstream, and infections of various organs outside the digestive system; however, most *Salmonella* infections are self-limiting ***(Dione et al, 2011).*** *Clostridium* is large, gram-positive, motile, obligate anaerobe spore-forming ,fermentative, catalase-negative, and generally motile. In the gastrointestinal systems of many animal species and humans, it appears as a normal commensal despite its widespread soil distribution. It only becomes a problem when there is a buildup of toxic exotoxins due to nutritional stress, injury, or parasitism ***(Brynestad and Granum , 2002).*** The purpose of this study was to detect and identify bacterial pathogens that cause diarrhoea in calves using bacteriological and biochemical techniques.

Material and methods

1-Sampling

faecal samples were obtained from diarrheic (cow-calf – buffalo calf) from four farms and sporadic cases from El-Sharkia governorate, including buffalo calves and cattle calves aged from seven days to one year. The samples were obtained early in the disease before antibiotic therapy was applied. The affected calves showed brown watery diarrhoea, sometimes tinged with blood. Farms that did not vaccinate pregnant dams against the clostridial disease before calving.

2- Isolation of bacterial species isolated from diarrheic calves

A -Isolation of *E. coli*

215 Faecal samples were inoculated into buffered peptone water (B.P.W))(Oxoid) and incubated at 37ºC for 24 hrs (pre-enriched medium); then, one ml from the pre-enrichment broth was transferred to 10 ml MacConky' broth (Oxoid) and incubated at 37ºC for 24 hrs; then cultivated, a loopful from each of incubated MacCkonkey's broth was streaked onto Eosin Methylene blue (EMB) (Oxoid) agar and incubated at37ºC for 18-24 hrs. Suspected small green fluorescence colonies were picked up, purified and streaked onto nutrient agar slopes and incubated at at37ºC for 18-24 hrs. Then preserved in refrigerator for further identification.

b-Isolation of *Salmonella*

From each sample, 10 a gram of faeces was mixed with 90 ml of pre-enrichment both (B.P.W) (Oxoid).

The prepared samples were incubated at 37ºC for 24 hrs. 1ml of pre-enrichment cultured broth was transferred to 10 ml of Rappaport vasiliadis (Oxoid) selective enrichment broth and incubated at 41ºC for 24hrs; a loopful from the incubated Rappaport Vassiliadis selective enrichment broth was streaked onto (XLD) (Oxoid) media and incubated at at37ºC for 18-24 hrs. After incubation, black colonies with red background were picked up. The purified colonies were streaked onto nutrient agar slants and preserved in a refrigerator for further identification.

c-Isolation of *C. perfringens*

The sample was inoculated into a tube of sterile, freshly prepared cooked meat medium(CMM) (Oxoid) and then incubated aerobically at 37ºC for 24 hrs in an anaerobic Jar using gas-generating kits. Consequently, a loopful of inoculated CMM broth was streaked on the surface of 5-10% sheep blood agar(Oxoid) containing neomycin sulphate in a concentration of 200µg/ml then the plate was incubated anaerobically at 37ºC for 24 hrs . Afterwards, the plate was examined for bacterial growth, and suspected colonies of *Clostredium* were picked up and examined for their morphological and biochemical identification.

3- Biochemical identification of isolates

Isolates were identified using culture characters, gram staining and biochemical reactions according to ***(Macfaddin ,2000)***

1-Oxidase test

the colony to be tested was transferred to an oxidase disc using a sterile toothpick. The culture was spread on the disc that developed deep blue or violet colour within 5 sec, indicating a positive reaction.

2 Catalase test

A loopful of bacterial growth was removed from the culture plate and smeared on a glass slide. A drop of 3 % (m/v) hydrogen peroxide was placed onto the bacterial cells, and the appearance of bubbles indicates a positive test.

3 Indole production test *(Abbott et al., 2003):*

A heavy loop of bacterial growth was sub-cultured onto a 5 ml tryptone water tube and incubated at 30°C for 24-48 hours. Then, 6-7 drops of Kovac's reagent were added, and the tubes were shaken. A positive result was developing a cherry red colour in the upper reagent layer on top of the medium. No colour development indicated a negative result.

4 Methyl-red and Vogues-Proskauer tests *(Abbott et al., 2003):*

A 10 ml tube of MR-VP medium was inoculated with two loopfuls of a pure, 4-6 hrs. Old peptone water culture of the organism under test. After incubation at 30°C for 48 hrs, one portion of the broth was tested with 5 drops of methyl red solution. A bright red colour's immediate appearance indicated a positive

result, while the formation of a yellow colour indicated a negative result. The second portion of the broth was used for the VP reaction by adding 3 ml of 5% (w/v) alcoholic α-naphthol solution (reagent A) and 3 ml of 40% (w/v) KOH solution (reagent B). With gentle shaking, the bright pink colour appeared within 20-30 minutes was positive; no colour appearance was negative.

5 Citrate utilization test *(Abbott et al., 2003):*

The test measures the ability of bacteria to utilize citrate as the sole source of carbon and ammonium dihydrogen phosphate ($NH_4H_2PO_4$) as the sole source of nitrogen. The utilization of $NH_4H_2PO_4$ to get N with the production of NH_3 increased the pH resulting in a change in the colour of bromothymol blue from green (pH= 6.9) to blue (pH above 7.6); the tested bacteria were inoculated aseptically on agar slant of ready-made Simmons citrate agar medium by stabbing into the butt and streaking on the surface of the slant with the help of a flame sterilized needle and incubated at 30°C for 24-48 hours. After incubation, if there is growth on the slant and colour changes from green to blue indicates, citrate utilization is positive.

6 Oxidation fermentation test *(Ahammed et al., 2016):*

in this test, the bacteria were grown aerobically and anaerobically separately in semisolid agar tubes containing glucose and bromocresol purple. If the bacteria can utilize glucose aerobically, the colour of the media changes from purple to yellow (Oxidative). If it uses glucose anaerobically (Fermentative), both tubes' colour changes from purple to yellow.

7 Sugar fermentation and gas production *(Abbott et al., 2003):*

purified colonies were inoculated into peptone water containing 1 % of the tested sugar (glucose- sucrose – mannose) using Andread's indicator. Durham's tubes were previously inserted into test tubes to collect gas. Incubation was done at 30 °C for 24-48 hours for the presence or absence of an acid colour change and gas formation. suppose the bacteria can utilize any three sugars (glucose, sucrose or lactose). In that case, acid is produced, which reduces the medium's pH and the colour of the media changes from purple to yellow.

8 Nitrate reduction test *(Sabur, 2006):*

The tested bacteria were grown in a broth medium containing nitrate (NO_3); if the bacteria can reduce nitrates, the broth acquires a red colour upon adding sulphanilic acid and α-naphthylamine. If red colour is not produced, it indicates that the bacteria do not construct either NO3 or the bacteria has a highly potent nitrate reductase enzyme that rapidly reduces NO_3.

9 H_2S production *(Ahammed et al., 2016):*

The bacteria were grown on a triple sugar iron agar slant (TSI agar slant)

(Oxoid). Suppose the bacteria utilize the inorganic sulphur (sodium thiosulphate) used in the medium. In that case, H_2S is produced, which combines with the ferrous sulphate in the medium to form black precipitates or ferrous sulphide resulting in a change in the colour of the butt to black. Besides utilizing inorganic sulphur.

10 Urea hydrolysis test *(Monir et al., 2017):*

The tested bacteria were grown on agar slants containing urea and phenol red if the bacteria can hydrolyze urea, the colour of the medium changes from yellow to pink.

Result

Prevalence of pathogenic bacteria

The incidence varied amongst farms, ranging from 0% to 27.9%. The buffalo calves were most affected than cattle calves with diarrhoea. The calves of age from seven days to three months were the most affected calves with diarrhoea. The most isolated bacteria were *E.coli* followed by *C.pefringens* and *Salmonella* spp. were the last isolated in this study in bacteriological testing through morphological characters and biochemical identification.

1-Identification *E. coli* isolates

A.Morphological character

E. coli isolated from samples on macCkongy agar as lactose fermenter gives pink colonies. Eosin methylene blue agar(EMB) was characterized by green metallic sheen large colonies .by Gram's stain were gram-negative rod-shaped bacteria moderate size motile, and non-spore-forming bacteria.

B. Biochemical character

E. coli isolated from samples biochemically were characterized by negative in the Vogues-Proskauer, citrate utilization test. The following tests give positive results; catalase, indole, and methyl red test, as shown in Table (1).

2- Identification of *Salmonella* isolates:

A.Morphological character

On macCkongy give, pale colonies as a non-lactose fermenter. *Salmonella* spp. red colonies with a black centre characterized isolated samples on XLD agar. Isolates were gram-negative moderate size with cell diameters between 0.7 and 1.5 μm, lengths from 2 to 5 μm, non-spore-forming and motile with peritrichous flagella bacteria.

B. Biochemical character

Isolated *Salmonella* biochemically was negative in indole test, Voges-Proskauer and methyl red, as shown in Table (2).

3-Identification of *C.perfringens* isolates:

A.Morphological character

C.perfringens isolated from samples on neomycin blood agar show a double zone of haemolysis. Isolates were a non-motile, Gram-positive bacillus rod-shaped and spore-forming bacteria.

B. Biochemical character

C.perfringens isolated from samples biochemically were characterized by

negative in the catalase and indole test. The following tests give positive results; H2S, nitrate reduction, and Urease, as shown in Table (3).

Table(1): *The biochemical characters of Escherichia coli*

Tests	Reactions
Gram staining	-ve
Catalase	+ve
Oxidase	-ve
Indole	+ve
Citrate	-ve
Methyle red	+ve
Voges proskaur (v.p)	-ve
H2S	-ve
Urease	-ve
TSI	+ve
Sugar fermentation : Glucose Lactose Maltose Sucrose Mannitol Dulictol	 +ve +ve +ve D +ve D

+ positive ,- negative ,D differs

Table (2) *Biochemical characters of Salmonella spp*

Cultures characteristics	***Salmonella***
Gram staining	-ve
Motility	Motile
Oxidase	-ve
Voges-Proskauer	-ve
Catalase	+ve
Citrate utilization	D
H_2Sproduction	+ve
Indole	-ve
Methyl Red	+ve
Fermentation of lactose	-ve
Fermentation of sucrose	-ve

Table (3) *Biochemical characters of C. perfringens*

Tests	reactions
Gram staining	+ve
Catalase test	-ve
Gelatin liquefaction	+ve
Glucose	+ve
Lactose	+ve
Sucrose	+ve
Galactose	-ve
Mannitol	-ve
Maltose	+ve
Xylose	-ve
Mannose	+ve
Indole	-ve
Nitrate reduction	+ve
H2S production	+ve
Urease	+ve
Lecithinase activity	+ve

Discussion

Neonatal calf diarrhoea is a disease of significant impact on the economic viability of cattle herds worldwide. The present study on 215 diarrheic neonatal calves showed variable degrees of diarrhoea which varied from mild to profuse watery faeces, it's colour differs from whitish-yellow to greenish and, in some cases, tinged with blood or mucous. Calves were suffering from dehydration, weakness, standing inability, and body temperature rise. This study intended to describe the prevalence of bacterial species associated with enteritis in neonatal calves ***(Martini,2008)***.

In this study, the prevalence rate of diarrhoea was higher in buffalo calves than in cow calves. These results differed from those ***(Malik et al ., 2012),*** who found no difference between the prevalence rates in cow and buffalo calves according to investigated farms. The highest rate of diarrhoea was observed (27.9%), and no diseased calves on another farm. This is due to differences in hygienic management conditions on each farm ***(El-Naker et al 2008).***

Bacteriological examination of faecal samples of diarrheic calves, the result obtained showed that *E.coli* was most isolated, followed by *C.perfringens*, and *Salmonella* spp. were the last isolated similar to those reported by ***(Selim et al ., 2003; Regobelo et al ., 2006 ; El-Naker et al 2008;Shehedi et al ., 2013; Saad , 2014; Ghareib et al ., 2015).*** *Ecoli* is normal commensal in calves, and under stress, factors turned to be pathogenic. *Clostridium perfringens* is generally found in the gastrointestinal tract of humans and animals. It is usually present in mixed infection I which the primary pathogen has passed the way by

damaging the tissue, causing anaerobiosis ***(Secasiu et al 1997)***

In conclusion

According to the findings of this study, *E.coli* is the most common pathogenic bacteria on farms. *C.perfringens* and *Salmonella* play a significant impact in diarrhoea and must be considered into consider when determining on preventative measures.

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الملخص العربي

إسهال العجول عبارة عن مرض متعدد العوامل يمكن أن يكون له آثار مالية شديدة على رفاهية الحيوان في كل من قطعان الألبان ولحوم البقر. المسببات الأمراض البكتيرية تعد السبب الرئيسي للإسهال الدموي في العجول ويسبب ارتفاع معدل الوفيات. هدفت هذه الدراسة إلى عزل البكتيريا المسببة للإسهال والتعرف البيوكيميائي للبكتيريا المعزولة. تم اختبار عينات البراز المأخوذة من عجول مصابة بالإسهال في هذه الدراسة ، بما في ذلك عجول الجاموس وعجول الماشية التي تتراوح أعمارها بين سبعة أيام وسنة. تفاوت معدل الإصابة بين المزارع حيث تراوحت بين 0٪ و 27.9٪. تأثرت عجول الجاموس بالإسهال أكثر من عجول الماشية. كانت العجول البالغة من العمر من سبعة أيام إلى ثلاثة أشهر هي العجول الأكثر إصابة بالإسهال. وكانت أكثر أنواع البكتيريا المعزولة هي *E.coli* تليها بكتريا *C.pefringens* و *Salmonella* spp. كانت اقلهم عزلا في هذه الدراسة في الاختبارات البكتريولوجية من خلال الصفات الظاهريه والتعرف البيوكيميائي.