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BACTERIAL SPECIES ISOLATED FROM DIARRHOEIC CALVES AND ITS ANTIBIOTIC SENSITIVITY PATTERN

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Abstract: This study was performed to identify various bacteria from feces of calves suffering from diarrhea, and to determine in vitro antimicrobial activity. Fecal samples were collected from 80 diarrheic calves and primarily tested for the presence of *Escherichia coli*, *Salmonella spp*. and *Staphylococcus aureus* using bacteriological examination, biochemical reactions. 60 bacterial isolates from the 80 fecal samples were identified using bacteriological and biochemical methods. *Escherichia coli* was considered to be the most frequent bacterium isolated numbering 22 (36.66%) followed by *Salmonella sp*. as the second most prevalent 11 (18.33%). Further isolates such as *Staphylococcus aureus* 8 (13.33%), *Enterococcus faecalis* 5 (8.33%) and *Shigella sp.* 3 (5.00%) were isolated and identified. Among the antibiotic sensitivity 55.0% were sensitive to Amikacin, 55% were sensitive to Ceftriaxone, 69.0% sensitive to Ciprofloxacin, 81.5% were sensitive to Kanamycin, and 75.5% were sensitive to Nalidixic acid (Table 4). Similarly majority of the bacterial isolates showed resistance to Ampicillin (75.0%), Amoxycillin (62.0%), Ceftriaxone (45.0%), Chloramphenicol (68.0%), Gentamicin (50.0%), Streptomycin (65.0%) and Tetracycline (74.0%).

Keywords: Bacteria, Calf diarrhea, Escherichia coli, Salmonella species - Antibiotic resistance.

Introduction

Calf diarrhea caused by bacterial infection has a bad effect on the dairy industry all over the world when calves are reared intensively. It involves significant economic loss for labor and capital, calf mortality, loss in calf value and veterinary costs (Pereira et al., 2011; de Verdier et al., 2012). The pre-weaning mortality of dairy calves was estimated at 10.8% level with diarrhea responsible for more than half of those deaths (Pereira et al., 2011; Wu et al., 2010). Diarrhea caused by a variety of bacteria has been recognized as one of the most public clinical problems for calves worldwide. Among these bacteria *Eschirechia coli* (*E. coli*) as "white scour", *Salmonella typhyimurium* (*S. typhimurium*), *Clostridium perfringens* (*C. perfringens*) and *Staphylococcus aureus* (*St. aureus*) are believed to be the major microbial causes of diarrhea in calves (Hemashenpagam et al., 2009; Abdullah et al., 2013; Cho YI et al., 2010).

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Antimicrobial agents are considered popular to fight diarrhea in calves. Nevertheless, their wide spectrum of activity, the emergence of microbial tolerance of different antimicrobial agents has become a well-known phenomenon, which represents a major concern (Hajipour et al., 2013). Resistance to antimicrobial agents was frequently occurred in Salmonella species and E. coli particularly in pre-weaned dairy calves (Izzo et al., 2011).

The frequent resistance of various microorganisms to the majority of antimicrobial agents is catching the attention of a great deal of awareness. The World Health Organization (WHO) commented on the severe threat formed by antibiotics-resistant bacteria in livestock and human health (Raffi et al., 2010). The frequent use of antibacterial agents has created the selective pressure to enhance the rising rates in antibiotic tolerance to different types of bacteria (El Zowalaty, 2012; Lee et al., 2013). Consequently, the microorganisms developed resistance against several types of antimicrobial agents to be one of the most important public health problems. In addition, the disadvantages of frequently used antimicrobial agents are not only the development of multiple drug resistance, but also adverse side effects (Huh and Kwon, 2011). As a result of antimicrobial resistance in the diarrhea of pre-weaned calves, there is an increasing attention in using alternative antimicrobial agent (Pereira et al., 2011; Hajipour et al., 2013). Consequently, there is an urgent need to find out an innovative approach and recognize new antimicrobial agents from natural and inorganic substances to develop the next generation of antimicrobial agents to control different microbial infections (Hajipour et al., 2013).

Materials and Methods

Collection of samples:

Rectal swabs were collected from (80) diarrheic calves using sterile cotton swabs and the samples were transferred directly to the laboratory in a separate clean sterile plastic bag, in an ice box and kept under complete aseptic condition without delay and subjected to bacterial culture, microscopic observation and antibiogram of *E.coli* and *Salmonella.*

Detection of bacterial fecal pathogens

Bacteriological examination:

1. **Isolation and identification of *E. coli*:** All samples were inoculated into tubes of freshly prepared nutrient broth and incubated aerobically at 37^{o}C over night, followed by subculturing onto MacConkey agar and Eosin methylene blue agar plates for 24-48 hours at 37^{o}C. Lactose positive colonies were confirmed as *E. coli* according to Gershwin (1990); Koneman *et al.*, (1992) and Quinn *et al.,* (1994). Suspected colonies grown were picked on

nutrient agar slopes and incubated at 37°C for 24 hours, then kept in refrigerator at 4°C for further identifications according to Edwards and Ewing (1972).

2. Isolation and identification of Salmonella

All samples were inoculated into tubes of Selenite-F and Tetrathionate broths and streaked out onto MacConkey and brilliant green agar after overnight incubation at 37°C. Suspected colonies were subjected to biochemical testing according to Adyin *et al.,* (2001) and Echeita *et al.,* (2002). Slide agglutination test was used for identification according to the Kauffmann-White Schema (Zahraei *et al.,* 2007; Nori and Thong, 2010). Fnally identification of *Salmonella typhimurium* was done according to Waltner-Toews *et al.,* (1986).

3. Isolation and identification of Staphylococcus

Isolation of presumptive S. aureus from the samples was performed using Mannitol salt agar (MSA). The plates were incubated aerobically at 37°C for 18 h – 24 h. Consequently, the characteristic S.aureus colonies that were yellow in color from MSA plate were further purified by sub-culturing onto MSA plates and the plates were incubated aerobically at 37°C for 18 h–24 h. These isolates were retained for further bacterial identification.

Microscopic Study by Staining Method

The microorganisms were isolated from suspected cases of fecal samples, and then stained with Gram's staining techniques (Merchant and Packer, 1967).

Identification of bacterial isolates by using specific biochemical tests

After culture characterization of *E. coli, Salmonella* spp. and *St. aureus* on different culture media as MacConkey agar, EMB agar, Blood agar, SS agar and Mannitol salt agar, all the isolates were identified by various biochemical tests including Catalase, Oxidase, Triple sugar iron (TSI) agar slant reaction, Voges–Proskauer (VP), Methyl-Red (MR), Indole, Motility indole and urease (MIU) test (Merchant and Packer, 1967).

Antibacterial Sensitivity Pattern of the Isolated Salmonella and E. coli

The overnight nutrient broth cultured *Salmonella* isolates were poured on SS agar and spread uniformly with the help of sterile glass spreader. Antibacterial discs were applied aseptically to the surface of the plate at an appropriate distance with the help of sterile forceps and incubated at 37°C for 24 hours, aerobically. Antibiotic sensitivity pattern of isolated *E. coli* and *Salmonella* were performed against 11 commonly used antibiotics belonging to different groups (Bauer et al., 1966) procured from HiMedia Pvt. Ltd. The antimicrobial agents tested were the following; Amikacin (AK) , Ampicillin (AMP), Ceftriaxone (CTR), Chloramphenicol, Ciprofloxacin (CIP), Gentamicin (GEN), Kanamycin, Nalidixic acid (NA),

Streptomycin (S) and Tetracycline (TE). Sensitivity of the isolated bacterial strains to selected antibiotics and sulfonamides was tested using the Kirby-Bauer disk diffusion method on Mueller-Hinton medium in accordance with accepted international norms (CLSI, 2011). The results were read and interpreted based on the diameter of the zone of inhibition, with the strains designated as resistant (R), of intermediate sensitivity (I) or sensitive (S).

Results

Characterization of bacterial fecal pathogens

Isolation and identification of E. coli, Salmonella spp., and St.aureus were carried out by using morphological, biochemical and genotypic characteristics. A total of 60 bacterial isolates were obtained from the 80 fecal samples. E. coli was considered the most frequent bacterium isolated (36.66%) followed by Salmonella sp. as the second most prevalent (18.33%) (Table 3). Further isolates such as Klebsiella spp. (18.33%), St. aureus (13.33%), Enterococcus fecalis (8.33%) and Shigella sp. (5.00%) were isolated respectively. In the current study, the sample found positive for *E. coli* gives a positive reaction to lactose fermentation on MacConkey agar plate, metallic green sheen colonies on EMB plates and yellowish green colonies on Brilliant Green Agar (BGA). The sample gives a positive reaction for Salmonella producing negative reaction to lactose fermentation on MacConkey agar plate, Opaque, transparent and pale colonies with black center were produced on SS agar and pink color colonies on Brilliant green agar. Additionally, the sample gives a positive reaction for St. aureus producing yellowish colonies on tryptose soya agar and hemolysis on blood agar and yellow colonies on Mannitol salt agar (Table 1).

The various isolates of *E. coli, Salmonella* spp. and *St. aureus* demonstrated identical results in various biochemical assays including sugar fermentation, Triple Sugar Iron (TSI) slant, Motility Indole Urease (MIU) test, Indole, Methyl Red and Voges-Proskauer Test (MR-VP), citrate utilization tests and Coagulase test. St. aureus produce acid but no gas by fermenting various sugars and gave positive reaction to coagulase, catalase and methyl red tests but negative reaction to Indole and Voges Proskeur test (Table 2).

Table 1: Identification of isolated microbial pathogens by cultural properties

Culture media utilized	Detection (Colony morphology)		
	E.coli	*Salmonella spp.*	*St.aureus*
Nutrient agar	Smooth, rounded, white to grayish colony with unusual putrid odor	Small, circular and smooth colonies	Growth of circular, small, smooth, convex, and golden yellowish colonies

Blood agar	Demonstrate haemolysis	Demonstrate haemolysis	Demonstrate haemolysis
Mac Conkey agar	Rosy pink lactose fermenter colonies	Colorless, pale, translucent colony	No growth (-)
Salmonella, Shigella (SS) agar	Pink color colony	Translucent colorless smooth colony with black center	No growth (-)
Mannitol salt agar	No growth (-)	No growth (-)	Yellow colonies
Eosin-Methylene Blue (EMB) agar	Moist circular colonies with dark centers, yellow green metallic sheen	Light purple to colorless	No growth (-)

Table 2: Biochemical characteristics of *E. coli* and *Salmonella* from diarrheic calves

isolated bacteria	Indole production test	Methyl–red test	Voges–Poskauer reaction	Citrate utilization test	MIU test	TSI Test	Hydrogen sulphide
E. coli	+	+	-	-	+	Butt–Y Slant–Y	-
Salmonella spp	-	+	-	-	-	Butt–Y Slant–R	+
Klebsiella spp	-	-	+	+	-	Butt–Y Slant–Y	-
St.aureus	-	+	+	+	-	Butt–R Slant–R	-
Enterococcus faecalis	-	-	+	-	-	Butt–R Slant–R	-
Shigella spp.	-	+	-	-	-	Butt–Y Slant–R	-

Table 3: Frequency of bacterial species isolated from calves suffering from diarrhea

Bacterial isolates	Number of isolates	Percentage (%) of isolates
Escherichia coli	22	36.66
Salmonella spp	11	18.33
Klebsiella spp	11	18.33
St. aureus	8	13.33
Enterococcus fecalis	5	8.33

Shigella spp	3	5.00
Total	60	99.98

Antibiotic sensitivity test of various isolates of *E. coli* and *Salmonella*

Among the antibiotic sensitivity 55.0% were sensitive to Amikacin, 55% were sensitive to Ceftriaxone, 69.0% sensitive to Ciprofloxacin, 81.5% were sensitive to Kanamycin, and 75.5% were sensitive to Nalidixic acid (Table 4). Similarly majority of the bacterial isolates showed resistance to Ampicillin (75.0%), Amoxycillin (62.0%), Ceftriaxone (45.0%), Chloramphenicol (68.0%), Gentamicin (50.0%), Streptomycin (65.0%) and Tetracycline (74.0%).

Table 4: Antibiotic sensitivity of bacterial species isolated from calves suffering from diarrhea

Antibiotics	Sensitive (%)	Intermediate (%)	Resistant (%)
Amikacin	55.00	2.50	42.50
Ampicillin	25.00	--	75.00
Amoxicillin	35.00	3.00	62.00
Ceftriaxone	55.00	--	45.00
Chloramphenicol	30.50	1.50	68.00
Ciprofloxacin	69.0	--	31.00
Gentamycin	47.75	2.25	50.00
Kanamycin	81.50	--	18.50
Naildixic acid	75.50	--	24.50
Streptomycin	35.00	--	65.00
Tetracycline	26.00	--	74.00

Discussion

Identification of bacterial fecal pathogens

In the current investigation, various types of bacteria (E. coli, Salmonella spp., Klebsiella spp., St. aureus, Enterococcus faecalis and Shigella spp.) were isolated from a total of 80 fecal specimens collected from form pre-weaned calves suffering from diarrhea. 22 samples were identified as E. coli which appeared as rosy pink lactose fermenter colonies on MCA plate, moist circular colonies with dark yellow centers, green metallic sheen on EMB plates and yellowish green colonies on the BGA. Eleven samples were identified phenotypically as Salmonella spp. because the organism on MCA plate gave colorless, pale, translucent colony with negative reaction to lactose fermentation, translucent colorless smooth colonies with black center on S-S agar, pink color colonies on BGA and pink colour colony with black center on DCA. Eight samples were found positive for St. aureus producing yellow pigmented golden colonies on Trypticsoy agar, yellow colour colony on Mannitol salt agar

and hemolysis on Blood agar. Dissimilarities in the colony shape appeared by the isolates may be as a result of losing or obtaining some properties by the transfer of host or choice of host tissue as noticed by Abdullah et al., (2013).

Various biochemical tests, including Indole, Voges-Poskauer, Methyl-red, Citrate, TSI, MIU and Coagulase were used to distinguish between different isolates of bacteria in this study. *E. coli* gave positive reactions to Indole and Methyl-red tests, but negative to Voges- Poskauer, Citrate and Coagulase tests. *St. aureus* gave positive reactions to Coagulase and Methyl red tests, however negative reaction to Indole, Voges Proskeur, Citrate and MIU tests was reported. Moreover, *Salmonella spp.* gave positive results to Methyl-red and MIU tests while negative to the other tests. To date, the real reasons for which the appearance of an identical results in biochemical reactions of the three groups of recognized isolates were not obvious. There is no doubt that nearly all bacterial isolates in the current investigation have several common genetic materials which might be responsible for the appearance of similar type of biochemical reaction as reported by Pandey et al., (1979) and Honda et al., (1982).

Antibiotic resistance and antimicrobial effect of various antibiotics

The present study is to evaluate the effect of various antibiotics on *E. coli, Salmonella* and *St. aureus* isolated from feces of calves suffering from diarrhea. Therefore, the antimicrobial sensitivity test of three various types of bacterial isolates to 11 different antimicrobial agents were studied. The sensitivity investigation demonstrated that the majority of the E. coli, Salmonella spp. and St.aureus were tolerant to ampicillin, tetracycline, amoxicillin and chloramphenicol. Similar results were obtained by Abdullah et al., 2013; Ahmad et al., 1986; Edrington et al., 2004; Nazir, (2007) who stated that calf isolates were resistant to ampicillin, amoxicillin, erythromycin and gentamicin (Nazir, 2007). These findings were somewhat different with the results of Guerra et al., 2006 and Joon and Kaura, (1993) who stated that most of the microorganisms isolated from calves suffering from diarrhea were highly susceptible to chloramphenicol and tetracycline and moderately sensitive to ampicillin and amoxicillin (Joon and Kaura, 1993). The variation in the susceptibility of antimicrobial agents against the fecal isolates may be due to the outcome of selection and also the random apply of antibiotic in various disease stages to different animal species. The appearance of multidrug-resistant bacteria is documented as an important problem for public health worldwide. Consequently, treatment is expensive and needs prolonged time.

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