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Detection of Virulence Genes and Antimicrobial Resistance of Bacterial isolates of Diarrhea in Newly Borne Buffalo Calves.

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ABSTRACT

Diarrhea in newly borne calves (Cattle or Buffalo) remains one of the important economically life threatening disease. The study was carried out on 120 buffalo calves, 80 suffered from diarrhea and 40 apparently healthy. They are aged from one day old till 2 months. The prevalence of Salmonellosis in examined calves was 15.8%. It was 21.25% in calves with diarrhea and 5% in apparently healthy ones. The prevalence of *E. coli* infection in examined calves was 15%. It was 18.75% in calves with diarrhea and 7.5% in apparently healthy ones. The virulence genes of Salmonella isolates including *invA* was detected in all isolates (100%), while *bcfC gene* was detected in 47% of the isolates. In contrast, *avrA* and *ssaQ* gene were identified in only 36% of the isolates. The *16 SrDNA ompA* and *fimH* genes were identified in all *E.coli* isolates and *Iss* gene was not detected in any isolates. The antimicrobial susceptibility was carried out on isolated *Salmonella and E. coli* strains using 14 different antibacterials. All *Salmonella* isolates were resistant to lincomycin, neomycin and gentamicin but all were susceptible to ciprofloxacin and streptomycin. *E. coli* isolates were resistant to neomycin, sulfamethoxazole + trimethoprim and nalidixic acid but all were susceptible to ciprofloxacin, gentamicin and streptomycin.

Keywords: *Salmonella, E.coli,* Buffalo calves, Virulence genes, Antimicrobial resistance.

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INTRODUCTION

Diarrhea in newly borne calves (Cattle or Buffalo) remains one of the important economically life threatening disease. The economic importance is related mainly to calf mortality and adverse effect on health status and longevity in the herd and the future productive performance in recovered buffalo calves [1]. The disease varied among different herds as it depends mainly on the managemental system including failure of passive transfer of immunity through Colostrum, bad sanitation measures and high degree of intensification [2,3]. Some buffalo farm records showed that calve losses can reach up to 22.2% in the first month of life and 23.7% of neonatal buffalo deaths are due to enteric disease [4].

Affected calves suffered clinically from diarrhea which varied from laxation to severe watery diarrhea with offensive odor, depression, dehydration, sunken eyes, and decrease milk intake to complete refuse of suckling. The affected animal may suffer from fever or not but at late stage of the disease in severe cases temperature may become subnormal at late stage of the disease and the animal suffer from recumbency and death. Many o f the clinical signs are associated with endotoxaemia. Sometimes calves also develop pneumoenteritis. [2,5].

The disease is multifactorial and studied as a syndrome as several pathogens are incriminated in causing of the disease including viruses as Rota and corona, bacterial as enteropathogenic E. coli and Salmonella and parasitic as Cryptosporidium and Toxocara vitullorum also management play an important role in the occurrence and spreading of the disease [6].

The incidence of the various etiologic agents differs according to the age of the buffalo calf. E. coli infection mainly occurred in young ages mainly in the first week of life while Salmonellosis occurred in older ages around 21 days. E. coli seems to be the major cause of diarrhoea in buffalo calves with an incidence of 54 to 58 % compared to 13 to 14 percent for Salmonellosis [7]. Other organisms may also be isolated from diarrheic calves as Clostridium perfringens types A, C and D, Pasteurella, Klebsiella, Proteus vulgaris and Citrobacter [8].

Escherichia coli is a usual microorganism of the intestinal aerobic flora in humans and animals Pathogenic strains of E. coli possess several virulence factors are involved in the pathogenesis of diarrhea. The major virulence factors are adhesions, enterotoxins and cytotoxis. Endotoxins cause fever, diarrhoea, haemorrhages and vascular thrombosis. The action of cytotoxic toxins as Vero cytotoxins (VT) and cytotoxic and necrotic factors (CNF), results in bloody diarrhoea, weakness, emaciation and anaemia .Pathogenic strains of E. coli may be shed by older calves or adult cattle with transmission to young calves by the fecal-oral route then propagated in diseased calves and shedding in very large numbers. [2,9].

Diarrhea can be the result of either increased secretion or decreased absorption. Bacteria such as ETEC and to some extent, Salmonella cause neonatal diarrhea by secreting enterotoxins that stimulate increased intestinal secretions.

Treatment of diarrhea is mainly based on stopping of suckling to one or two suckling, administration of fluids and electrolytes for rehydration of the diseased animals, intestinal protectants to decrease the fluid losses and antibiotics to compete the bacterial infections. Italy in the last years showed an increase in antimicrobial resistance for the *E. coli* strains while *Salmonella* spp. showed greater susceptibility patterns [10].

The objective of this study was to determine the prevalence of *Salmonella* and *E. coli* infection among the diarrheic and apparently healthy buffalo calves, detect the virulence -related genes among *Salmonella* and E. coli isolates and to evaluate the presence of antimicrobial resistance.

MATERIALS AND METHODS

The study was carried out on 120 buffalo calves, 40 calves were apparent healthy and 80 calves suffered from diarrhea. They were 1day to 2 months old. Diseased calves suffered from diarrhea which ranged from laxation till watery diarrhea with or without offensive odor as in fig. 1, some of them suffered from dehydration with sunken eyes. The body temperature of diseased calves ranged from 36 C (Subnormal) till 41 C (fever) in some cases.

Bacteriological examination

Sample

One hundred and twenty fecal swabs collected from apparent healthy (40swabs) and diarrheic calves (80 swabs) in Giza governorate. The collected swabs were transported immediately to the laboratory of the Department of Bacteriology and Mycology, faculty of Veterinary Medicine, Cairo University, in ice-cold containers and processed within 6 h of collection.

Isolation and identification:

All fecal swabs were processed for isolation and identification of bacterial causes of calf scour. The samples were cultivated according to theISO-6579-1993 standards [11] for isolation of *Salmonella* species and cultivated onto MacConkey agar medium for identification of other enteric types. The specimen also cultivated anaerobically for cultivation of *Clostridium* species. Presumptive Gram negative bacilli were inoculated onto micro-tubes of API 20E strips (bioMe´rieux, Marcy L'E´ toile, France) in accordance with the manufacturers' instructions.The bacteria were identified using the databaseAPI LAB Plus version 3.2.2 (bioMe´rieux), while other suspected colonywere subjected to detect biochemical and serological activities according to [12,13].

PCR identification and virulotyping

The isolates were further screened by PCR for identification and detection of virulence determinants using the primers mentioned in Table (1). Screening of PCR products by agarose gel electrophoresis in comparison with 100 bp– 1.5kb DNA ladder (Qiagen) was done.

Antimicrobial susceptibility testing using the disc diffusion method

The antimicrobial resistance profiles of the isolates were determined using discs impregnated with a range of antibiotics at fixed concentrations (Becton, Dickinson & Co.). The following antimicrobials were chosen on the basis of their common use in treating and preventing *Salmonella* and *E.coli* spp. infection including: ampicillin (10 mg), amoxicillin (20 mg), gentamicin (10 mg), neomycin (30 µg), streptomycin (10 mg), lincomycin (30 µg), chloramphenicol (30 mg), colistin (10 mg), tetracycline (30 mg), trimethoprim (5 µg), sulfamethoxazole + trimethoprim (23.75 mg + 1.75 mg). In addition, resistance was determined against three broad-spectrum fluoroquinolones: ciprofloxacin (5 µg), norfloxacin (5 µg) and nalidixic acid (30 mg).

The isolate was inoculated onto a Muller–Hinton agar (Oxoid) plate and antibiotic discs were placed on the surface of the agar (six discs per plate). The plates were incubated for 24 h to 48 h at 37°C. After incubation, the diameter of the halos was measured to assess resistance or susceptibility according to the interpretation criteria for *Escherichia coli (E. coli)* (ATCC No. 25922) established by the Clinical Laboratory Standards Institute [14]. Multidrug resistance is defined as resistance to two or more antibiotics belonging to different antibiotic classes [15].

RESULTS

Prevalence of microbial isolates

Microbiological examination of the fecal swabs revealed lactose and non-lactose fermenter colonies on MacConkey. The colonies were identified as Gram negative bacilli, catalase positive and oxidase negative. Biotyping results were *Salmonella* and *E.coli* species. Results indicated 37 isolates were identified as *Salmonella* (19 isolates) and *E. coli* (18 isolates) from both diarrheic and apparently healthy calves as in table 2 and table 3. No anaerobic microbes were detected.

PCR and Virulotyping

All isolates were screened using PCR analysis for detection and the presence or absence of 3 selected virulence genes each detected type (Table 4).The genes *invA* (carried by Salmonella pathogenicity islands [SPIs]) were detected in nineteen isolates,while*bcfC* (fimbria-related) were detected in nine isolates. In

contrast, *avrA* (located in SPI-1) and *ssaQ* (secretion systemapparatus protein) were identified in seven isolates. The genes*16 Sr DNA* were identified in all *E.coli* isolates. The genes *ompA* and *fimH*were positive in all isolates, while *Iss* gene was negative in all isolates.

Distribution of resistance to individual antimicrobial agents

Frequent resistance to the antimicrobials tested was evident in all isolates of *salmonella* and *E.coli* (Fig. 2). In particular, all *salmonella* isolates were resistant to lincomycin, neomycin and gentamicin but all were susceptible to ciprofloxacin and streptomycin; resistance patterns to the other antimicrobials exhibited great diversity. *E.coli* isolates were resistant to neomycin, sulfamethoxazole + trimethoprim and nalidixic acid but all were susceptible to ciprofloxacin, gentamicin and streptomycin.

Association of antimicrobial resistance phenotype with virulence-associated genes

Analysis of the presence of the virulence-associated genes in the tested isolates is shown in Table 5. Detailed analysis revealed associations of resistance/susceptibility phenotypes with potential virulence genes. The association of genes *invA, bcfC, avrA*and*ssaQ* with particular antimicrobial resistance phenotypes (lincomycin, neomycin and gentamicin) has been recorded in *Salmonella*. The presence of *16 SrDNA ,ompA*and *fimH*was associated with resistance to neomycin, sulfamethoxazole + trimethoprim and nalidixic acid was recorded in *E.coli*.

DISCUSSION

Diarrhea in newly borne buffalo calves is considered as one of the most economically important life threatening diseases. The economic importance is related mainly to calf mortality and adverse effect on health status and longevity in the herd and the future productive performance in recovered buffalo calves [1]. Bacterial diseases including Salmonellosis and Colibacillosis are considered as important infectious causes of diarrhea [3,7,16]. The study focused on the prevalence of *E. coli* and *Salmonella* in healthy and diarrheic newly borne buffalo calves aged from 1 day to 2 months old. Studying the virulence factors associated with the isolated strains of salmonella and E. coli and the antimicrobial susceptibility to different antibacterials. The study was carried out on 120 fecal swabs collected from newly borne buffalo calves (1day to 2 months), 80 calves were suffered from diarrhea and 40 were apparently healthy.

The prevalence of Salmonellosis in examined calves was 15.8%. It was 21.25% in calves with diarrhea and 5% in apparently healthy ones and these results were relatively agreed with [17]. It's 3.3% in calves with diarrhea within the first 2 weeks of life but not detected in apparently healthy calves in this age. The prevalence in age group of 2-4 weeks was 25% in calves suffered from diarrhea and 10% in apparently healthy calves. In the group aged 4-6 weeks the prevalence of Salmonellosis was 35% in calves with diarrhea and not detected in apparently healthy ones. In the last group aged 6-8 weeks old the prevalence was 40% in diarrheic calves and 10% in apparently healthy calves. The highest prevalence of Salmonellosis was observed among the age group 6-8 weeks followed by 4-6 weeks then 2-4 weeks and the lowest prevalence is found in the group up to 2 weeks old and these results agree with the results reported by [18] and results obtained from Sharkia samples examined by [19] but it's higher than the total results of all governorates reported by [19,20]. Salmonella was isolated from 7.5% of apparently healthy calves, these calves may be recovered or subclinical carriers and can shed the organism intermittently for variable periods of time and depending on the degree of infection and may act as a source of infection for other healthy calves as mentioned by [6].

The prevalence of *E. coli* infection in examined calves was 15%. It was 18.75% in calves with diarrhea and 7.5% in apparently healthy ones it was lower than that reported by [17,21] who reported 35%. It's 26.7% in calves with diarrhea within the first 2 weeks of life and detected within 10% in apparently healthy calves in this age. The prevalence in age group of 2-4 weeks was 25% in calves suffered from diarrhea and 20% in apparently healthy calves. In the group aged 4-6 weeks the prevalence was 10% in calves with diarrhea and not detected in apparently healthy ones. In the last group aged 6-8 weeks old *E. coli* infection was not detected in either diarrheic or apparently healthy calves and these results were comparable to the results obtained by [22] and lower than those reported by [23]. The highest prevalence was reported in calves lower than 2 weeks old and this is the most important age of *E. coli* infection as mentioned by Recently PCR technique is used as an important tool for confirmation of the diagnosis of the pathogenic infectious agents

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through detection of certain virulent genes [19]. In the present study all *Salmonella* isolates were screened using PCR analysis for detection of the presence or absence of the virulence genes (*invA, bcfC*, *avrA*, *ssaQ*). The *invA* gene which is responsible for invasion of *Salmonella* was detected in all isolates (100%) and this agreed with [18], while *bcfC gene* (Bovine colonization factor) was detected in nine isolates (47%). In contrast, *avrA* (Inhibits the pro-inflammatory) and *ssaQ* (Secretion system apparatus protein) gene were identified in only seven isolates (36%).

Table I: Detection and virulence factor primers, including nucleotide sequences, polymerase chain reaction conditions and references

Gene designation		Oligonucleotide sequences (5'-3')	PCR conditions			Product size (bp)	Reference
			Denaturing	Annealing	Extension		
Salmonella	*invA*	gtgaaattatcgccacgttcgggcaa tcatcgcaccgtcaaaggaac g	°72C for30 s	64°C for 30 s	94°C for 60 s	284	**[24]**
	avrA	Cctgtattgttgagcgtctgg agaagagcttcgttgaatgtc c	°95C for30 s	58°C for 30 s	°72C for30 s	422	**[25]**
	ssaQ	gaa tag cgaatgaagagcgtcgtc c cat cgtgttatcctctgt cag c				455	
	bcfC	accagagacattgccttc c ttctgctcgccgctattc g	°72C for30 s (b)	53°C for 30 s	94°C for 30 s	467	
E.coli	*16 SrDNA ECO*	Gacctcggtttagttcacaga cacacgctgacgctgacc a	94°for30 s	60°C for 1 minute	68°C for 2 min	585	**[26]**
	ompA	cttgcg gag gcttgtctgag agg cat tgctgggtaaggaa	94° for 1 min and 30 s	55° for 1 min and 30 s	72° for 2 min	1425	**[27]**
	lss	gtggcgaaa act agtaaaacagt cgcctcggggtg gat aa				737	
	fimH	caaaacctggtcgtg gat ct ttgccgttaatcccagactc				670	**[28]**

Table 2: Prevalence of *Salmonella* and *E. coli* isolates

Calves	*Salmonella* (19)		*E. coli* (18)	
	No.	%	No.	%
Apparently healthy (40)	2	5 %	3	7.5%
Diarrheic calves (80)	17	21.25%	15	18.75%
Total (120)	19	15.8%	18	15 %

Table 3: Prevalence of *Salmonella* and *E. coli* in different age groups.

Calves		*Salmonella* (19)		*E. coli* (18)	
		No.	%	No.	%
1 day -2 weeks (40)	Diarrheic calves (30)	1	3.3%	8	26.7%
	Apparently healthy (10)	0	0	1	10%
2 weeks – 4 weeks (30)	Diarrheic calves (20)	5	25%	5	25%
	Apparently healthy (10)	1	10%	2	20%
4 weeks – 6 weeks (30)	Diarrheic calves (20)	7	35%	2	10%
	Apparently healthy (10)	0	0	0	0
6 weeks – 8 weeks (20)	Diarrheic calves (10)	4	40%	0	0
	Apparently healthy (10)	1	10%	0	0

Table 4: PCR and Virulotyping for *Salmonella* and *E.coli*

	invA	*avrA*	*ssaQ*	*bcfC*	*16 SrDNA*	*ompA*	*lss*	*fimH*
Salmonella	19 (100%)	7 (36%)	7 (36%)	9 (47%)	------	----	----	-----
E.coli	-----	-----	-----	-----	18 (100%)	18 (100%)	0 (0%)	18 (100%)

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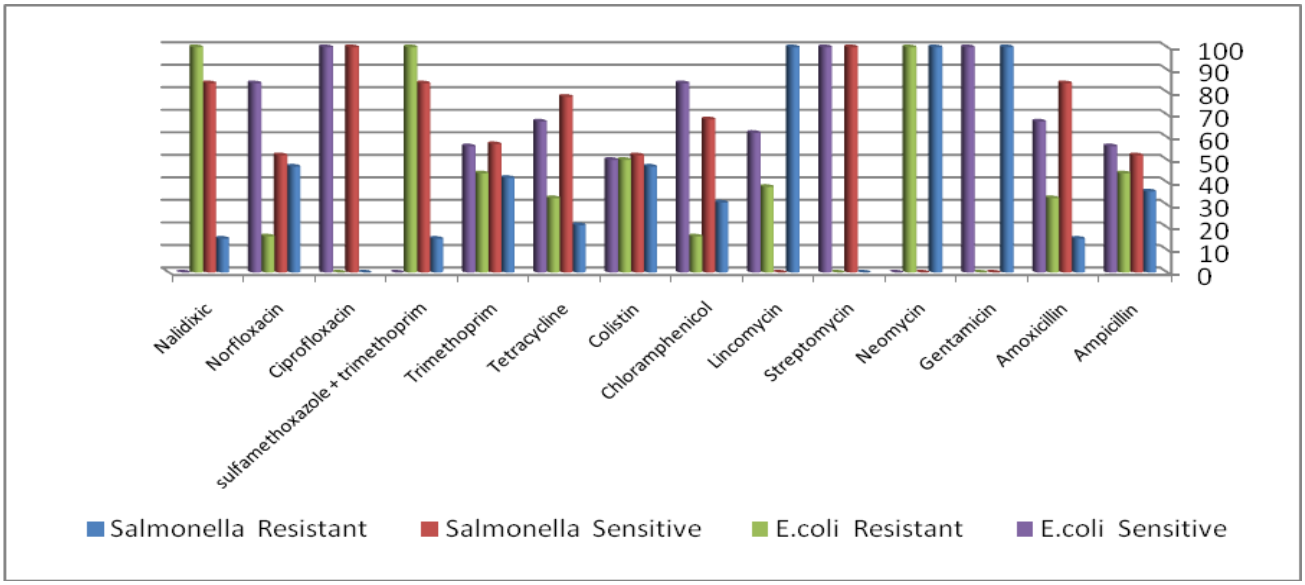
Table 5: Distribution of virulence gene combinations and antibiotic resistance phenotypes in *Salmonella and E.coli*

Isolates	Virulence gene combination	Resistance phenotype
Salmonella	*invA, bcfC, avrA, ssaQ*	lincomycin, neomycin and gentamicin
	invA, bcfC, avrA	Ampicillin, trimethoprim, chloramphenicol
	invA, bcfC	Colistin, nalidixic
	invA,avrA	Norfloxacin
E.coli	*16 SrDNA, ompA,fimH*	neomycin, sulfamethoxazole + trimethoprim, and nalidixic acid

Figure 1: Buffalo Calf showing Diarrhea

Figure 2: Antimicrobial resistance of *Salmonella* and *E.coli* isolates to different antibacterials:



All *E. coli* isolates were screened using PCR analysis for detection and the presence or absence of the virulence genes. The *16 SrDNA* gene was identified in all *E.coli* isolates (100%). The genes of *ompA* and *fimH* were positive in all isolates (100%), while *Iss* gene was not detected in any isolates.

The antimicrobial susceptibility was carried out on isolated *Salmonella and E. coli* strains using 14 different antibacterials. All *Salmonella* isolates were resistant to lincomycin, neomycin and gentamicin but all were susceptible to ciprofloxacin and streptomycin. Antibacterial susceptibility patterns to the other antimicrobials exhibited great diversity. *Salmonella* isolates have variable susceptibility to the following

antibacterials, amoxicillin, nalidexic acid and sulfamethoxazole with trimethoprim (84%) followed by tetracycline (78%), chloramphenicol (68%), trimethoprim (57%) while ampicillin, colistin and norfloxacin were 52%. *E. coli* isolates were resistant to neomycin, sulfamethoxazole + trimethoprim and nalidixic acid but all were susceptible to ciprofloxacin, gentamicin and streptomycin. They have variable susceptibility to the following antibacterials, norfloxacin and Chloramphenicol (84%), while amoxicillin and tetracycline were 67%, trimethoprim and ampicillin were 56% and colistin 50%. These results were comparable with the results obtained with [21].

The study revealed that there is association between virulence-associated genes of the tested isolates and antimicrobial resistance. In salmonella isolates the association was reported as presence of *Salmonella* virulent genes *invA, bcfC, avrA* and *ssaQ* were associated with antimicrobial resistance to lincomycin, neomycin and gentamicin, presence of *invA, bcfC* and *avrA* genes in the isolates were associated with resitence to Ampicillin, trimethoprim, chloramphenicol, presence of *invA* and *bcfC* were associated with resistence to Colistin, nalidixic and presence of *invA and avrA* were associated with resistence to norfloxacin. In E. coli isolates, the presence of *16 SrDNA ,ompA* and *fimH* genes were associated with resistance to neomycin, sulfamethoxazole + trimethoprim and nalidixic.

The appearance of antimicrobial resistance in zoonotic bacteria as *Salmonella* and *E. coli* is very important as a public health hazard as it increases the risk of treatment failure. Antibiotic-resistance and virulence determinants may be present in the same plasmid, which may be selected by antibiotic pressure resulting in increased systemic infections.

CONCLUSION

Diarrhea in newly borne buffalo calves still one of the most important causes of deaths. Salmonella and E. coli infections remain the major bacterial diseases causing diarrhea in newly borne calves. Antimicrobial resistance was developed to *Salmonella* and *E. coli* isolates against different antibacterials. Prevention of diarrhea requires development of prophylaxis protocols including the use of specific vaccines for the effective control of Salmonellosis and Colibacillosis in water buffalo calves.

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