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SOME BACTERIAL CAUSES RESPONSIBLE FOR DISEASES AND MORTALITIES IN CATTLE AND BUFFALO

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ABSTRACT

The high mortality rate in cattle and buffalo is a concern, especially in developing countries, such as Egypt. Under conditions of global change in climatic conditions, has caused the activation of some opportunistic microbes, causing deaths in cattle and buffalo farms. As a result of environmental stressors which lead to the activation of some bacterial agents which is the main reason for the increase in mortality in this article, we reported special spots on some bacterial agents causing sudden death in cattle and buffalo. Fecal and nasal discharge samples were collected for bacteriological examination from (60 diseased cattle and buffalo showing clinical signs of respiratory and diarrheic symptoms 30 from each of different ages and sexes (first group) and 40, slaughtered or dead animals (cattle and buffalo) from ruminal contents, tissue samples from liver, spleen, and lung 20 from each (second group). We collected samples from many locations at Assiut Governorate and were sent to the laboratory. The results of the first group indicated that (50) 30 cattle and 20 buffalo were positive for bacterial infection however 10 showed negative results of bacterial infection in buffalo. The incidence of bacterial infection in the first group was 83.5%, whereas the second group indicated that 100% (40) (20 cattle and 20 buffalo) were positive for bacterial infection. Bacteriological investigations of first group revealed that the isolated organisms were: *Clostridium perfringens* (46.7%), *Pasteurella multocida* (33.3%), *Escherichia coli* (13.3%), *Salmonella sp.*(6.7%) in cattle but in buffalo recorded that *Clostridium perfringens* (33.3%), *Pasteurella multocida* (23.3%), *Escherichia coli* (6.7%), *Salmonella sp.*(3.3%) , On other hand, bacteriological investigations of second group revealed that the isolated organisms were: *Clostridium perfringens* (50%), *Pasteurella multocida* (30%), *Escherichia coli* (15%) and, *Salmonella sp.*(5%) in cattle whereas in buffalo showed that *Clostridium perfringens* (40%), *Pasteurella multocida* (25%), *Escherichia coli* (25%), *Salmonella sp.*(10%), A total of 42 isolates of *Clostridium perfringens* isolated from first and second group 12 isolates examined for type of toxin by using of Multiscreen Ag ELISA Enterotoxemia The main often occurring toxin type was type A (buffaloes: 5/50; cows: 7/50) Out of 14 isolates of *E coli* from first and second group examined samples were serologically positive to O111, O104, O26, O113, O91 ,O103 and O126. PCR results showed that ten strains from 28 isolates, from the first and second group, were isolates and some of them have Kmt1, BlaROB1, and *tetH* genes. Antibiogram was applied upon the isolated bacterial pathogens and found that Ofloxacin – oxytetracycline – Aztreonam (*Clostridium perfringens*) Gentamycin, Ceftriaxone, and Norfloxacin (*Pasteurella multocida*) garamycin and oxytetracycline (*Escherichia coli*) and ciprofloxacin and ceftriaxone were the most effective antibiotic against *salmonella sp.* were the drug of choice for treatment of infected animals.

***keywords*:** enteritis; ceftriaxone; toxin; host microbiota *Clostridium perifr*; *Escherichia coli*

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INTRODUCTION

Mortality in cattle and buffaloes represents a major problem in Egypt and is difficult to diagnose due to its multiple causes Selim *et al.* (2017). Hemorrhagic septicemia is a bacterial disease that has a significant impact on mortality rates in cattle and buffalo farms in Asia, Africa, and the Middle East Annas *et al.* (2015). It also occasionally occurs and has caused fatalities in other animals, such as the endangered saiga antelope. Traditional diagnostic methods are used, such as serotyping, biotyping, and antibiotic determination, in addition to molecular methods and characterization, along with rapid epidemiological investigations of the spread of hemorrhagic septicemia (Yeruham, *et al.,* 2016).

The World Organization for Animal Health has recorded that classic hemorrhagic septicemia is caused by *Pasteurella multocida* serotypes B:2 and E:2. Serotype B:2 has been identified in most areas where the disease is endemic, and serotype E:2 has only been found. In Africa. Septicemic pasteurellosis, clinically similar to hemorrhagic sepsis, is caused by a wide range of other serotypes of *P multocida*. Statistics also indicate that 5% of Egyptian buffaloes and healthy livestock are colonized by small numbers of *P. multocida* serotype B:2 or E:2 (Chandranaik *et al.,* 2016). There are many common stressors associated with the spread of hemorrhagic septicemia, such as high temperature and humidity, concurrent infection (blood parasites or foot-and-mouth disease), malnutrition, and stress. The disease is more prevalent during the rainy season. The methods of infection often occur through contact with infected oral or nasal secretions from animals carrying the disease or animals carrying the microbe, or through consuming contaminated feed or water. Symptoms of the disease can appear 1-3 days after infection, and death can occur within 8-24 hours after the signs and symptoms appear. Német *et al.* (2017).

Hemorrhagic septicemia affects older and younger calves in areas where the disease is endemic. Mortality rates are variable and not constant due to many factors including age and sex. In non-endemic areas, epizootics can occur with high morbidity and mortality rates that can reach 100%. Buffalo tends to have a higher morbidity with more severe clinical disease than cattle. It can stimulate recovery and the immune system against homologous and often heterologous strains of *P. multocida*, and some of these animals become carriers and a source of infection in future outbreaks Annas *et al.,* (2014) (*Clostridium perfringens* has been induced necrotic diseases such as myonecrosis, acute watery diarrhea, and enteritis, which lead to death and associated with abundant life loss and high mortality rates and economic problems in cattle and buffalo farms. *Clostridium perfringens* is a Gram-positive, anaerobic bacterium that causes a wide range of diseases in animals. It is widespread in the environment (e.g., in soil and sewage) and is commonly present in the gastrointestinal tract of animals, especially in cattle and buffalo Prescott *et al.* (2016).

BioNumbers. (2022) reported that *Clostridium perfringens* was provisionally identified as immobile black colonies, which converted nitrate to nitrite, produced acid and gas from lactose, and liquefied gelatin within 48 h. *Clostridium perfringens* is divided into five toxin types, designated A to E, which are identified based on the main toxins they produce. These toxins cause symptoms and syndromes attributed to each type. Each type of toxin is associated with specific intestinal infections in different animal species (Ashgan et al., 2013; Ohtani and Shimizu 2016). Previous investigators (Baums, *et al.,* 2004). Daneshmand *et al.* (2022) reported *Clostridium perfringens* causing infection rates in live cattle with diarrhea (78.9%) and 94.4% in dead cattle examined immediately postmortem. Detection of *C. perfringens* toxin species is critical to ensuring understanding of *Clostridium perfringens* infection and may be useful in developing effective disease control (Das *et al.,* 2012).

While *C. perfringens* is often present in the intestines of healthy cattle and buffalo, so that a simple culture is unreliable in diagnosing disease caused by *C. perfringens*. Therefore, symptoms are matched to anatomy, and in some cases, toxins are identified, to obtain a true diagnosis (Smith 2014; Theoret *et al.* 2015). The method for typing *C. perfringens* toxins is based on neutralizing the pathogenic effect of each major toxin produced. This is done by using appropriate antisera to neutralize the toxin (Ammar *et al.* 2008; Gohari *et al.* 2015). The success in inducing various diseases comes, in part, from the ability of *C. perfringens* to produce more than two dozen disease-causing toxins and enzymes in cattle and buffalo. *C. perfringens* strains are classified into seven toxin types (A to G) based on their ability to produce different combinations of six major toxins, namely alpha (CPA), beta (CPB), epsilon (ETX), and ota (ITX). enterotoxin B (CPE) and enterotoxin-like toxin B (NetB). Alpha toxins (CPA or PLC) are produced by all *C. perfringens* strains, although toxin type A strains typically produce higher amounts than other toxins.

Fasina and Lilleho (2019) recorded that CPA suppresses the immune response by various methods as restraining leukocytes from entering the infected tissues [Lehman and Segal (2020)] and decrease the blood supply to infected sites by triggering vasoconstriction, thrombosis, and platelet aggregation [Bortoluzzi *et al.* (2019). CPA is a classic example of a toxin that modifies cell membranes by enzymatic activity. CPE is an important virulence factor for *C. perfringens* type A gastrointestinal disease in cattle and buffalo. This toxin is a zinc-dependent phospholipase C which degrades phosphatidyl choline and sphingomyelin, both components of the eukaryotic cell membranes (Bansal *et al.,* 2020) causing damage on the membrane of erythrocytes and other cells from many animal species. The net result of this action is cell lysis, by degradation of membrane phospholipids (Agus *et al.,* 2021). CPA activates several other membranes and internal cell mechanisms that lead to hemolysis. In addition, CPA activates the arachidonic cascade resulting in the formation of thromboxane, leukotrienes and prostaglandins, which activate the inflammation cascade and produce vasoconstriction (Khalique *et al.,* 2020)

C. perfringens is often cultured at 37 ◦C anaerobically with a lot of media as cooked meat medium, Duncan-Strong sporulation medium, Fluid thioglycolate medium (FTG), Tryptic soy broth (TSB) as recorded by Gong *et al.,* (2021) also, Tryptose-sulfite-cycloserine (TSC) agar (Shrestha *et al.,* (2019) and TSA with egg yolk emulsion or 5% sheep blood Lee, *et al.* (2019) and Lu, *et al.,* (2020).

E coli and *salmonella species* were represented as a cause of high mortality rates in cattle and buffalo especially in newly born calves. Calf mortality has been reported to be very high in cow and buffalo neonates Kopic and Geibel (2010). This mortality has mostly been attributed to infectious agents, i.e. (viral causes) a rotavirus, coronavirus, (bacterial causes) as enteropathogenic *Escherichia coli, salmonella species* and (parasitic causes) as cryptosporidium. Other important causes of calf mortality include immunodeficiency, seasonal effects parity of the dam, difficult parturition, sex and birth weight of the neonate and faulty management conditions. Of the infectious agents Moredo *et al.,* (2015), *E. coli* and *salmonella species* are mainly involved in the causation of neonatal calf diarrhea which leads to high mortality and morbidity in young calves. *E. coli* mainly plays its role up to the second week of life while *salmonella species* up to third week. Pneumonia causes great economic losses in neonatal calves. Dubreuil, (2012)

Enterotoxigenic *Escherichia coli*

Enterotoxigenic *Escherichia coli* produces severe diarrhea in calves mainly during the

first two weeks of life (Okello *et al.,* (2015)) and even some reports are available that the highest frequency of *E. coli* occurs in calves younger than 3 days old (Abraham *et al.,* (2012). *E. coli* produces enterotoxic and septicemic colibacillosis in young calves (Luo *et al.,* (2015). In enterotoxic colibacillosis, the pathogenic *E. coli* adheres to the mucosa and proliferate in the lumen of intestine, producing a potent enterotoxin, which stimulate excessive secretion of fluid from intestinal mucosa (Werneburg *et al.,* 2015). This loss of fluid causes the principal sign (diarrhea) and often leads to dehydration and high rate of death in the neonatal calves (McLamb *et al.,* (2013)). In septicemic colibacillosis, the organisms invade the host possibly through the oral cavity, respiratory system, pharynx, or umbilicus and produce endotoxin that apparently causes the lesions. Unless the enterotoxic form occurs simultaneously, the bacteria do not reach to the small intestine, thus diarrhea or intestinal lesions do not occur (Coddens *et al.,* 2013), Moonens *et al.,* (2015). Calves that are deficient in immunoglobulins are mostly susceptible to this form of colibacillosis (Gao *et al.,* 2013). The signs and lesions are typical of bacterial arthritis, polyserositis, meningitis and pyelonephritis with bacterial emboli and necrotizing, purulent, or fibrinous exudate (Mortezaei *et al.,* 2015). The incidence of *E. coli* in calf diarrhea varies very widely. Different strains of *E. coli* are prevalent in diarrheic calves, mostly K 99+ antigen was possessed by *E. coli* found involved in neonatal calf diarrhea (Bihannic *et al.* 2014), Madar Johansson *et al.* (2014).

Salmonella

Salmonella infections are most frequent and of great concern to young animals. These rod-shaped, gram-negative organisms are usually motile and produce gastroenteritis with nausea, vomiting, cramps, and diarrhea, salmonella in neonatal calves (28 days old) produces diarrhea in 1 to 12 percent calves (Tables 2 and 3) and morbidity up to 20 percent (Goetstouwers *et al.,* 2014)).

Valat *et al.,* (2014). Reported that *Escherichia coli* and *salmonella species* could be isolated from diarrheic samples as well from the suspected materials like nasal discharge or lung tissue from pneumonic lungs of calves, by following standard techniques of isolation

MATERIALS AND METHODS

1-Samples tested: One hundred blood and fecal samples (60 from diseased cattle and buffalo which showed clinical signs (anorexia, depression, diarrhea, cough fever, shortness of breath and chest pain) and 40 from recently died cattle and buffalo (intestinal organs (small intestine, colon, and cecum with typical lesions (thin intestinal wall, filled with gas, confluent mucosal necrosis of the small intestine, and depressed ulcers in the mucosal surface) and lung tissues were collected. Pneumonia is the most common type of infection, although tracheobronchitis, emphysema, and lung abscesses may also occur

2. Isolation and identification

2.1. Isolation and identification of *Clostridium perfringens* The fecal samples were streaked onto blood agar plates containing 7% sheep blood and incubated in an anaerobic chamber at 37°C for 48 h. Colonies that showed dual hemolytic zones were picked and subcultured in Tryptose Sulfite Cycloserine agar (TSC agar, Oxoid, Merck) for purification and were grown in fluid thioglycolate (FTG, Merck) for toxin production. For the intestinal organs, samples were taken by scrubbing the intestinal inner wall of the affected animal with cotton swabs and then processed in the same way as fecal samples. The identity of the isolates was confirmed by their colonial and microscopical morphology, hemolytic pattern, Gram staining, and biochemical tests as previously described [Quinn *et al.,*1994].

2. 2. Isolation and identification of *Pasteurella Multocida*

2.2.1 Easy and direct diagnosis

Blood smears from animals revealed bipolar organisms suggestive of Pasteurellosis by taking direct smear from blood and spread on a glass slide then stained by gram stain and examined microscopically for the bipolarity of *Pasteruella*. However, the blood smears from live in-contact animals revealed only very few bipolar organisms.
Postmortem examination revealed petechial hemorrhages in the heart, pericardial effusion, pale necrotic areas in the liver and congested, edematous lungs.

2.2.2 Isolation on blood agar,

After 24 hours post-inoculation, convex, mucoid, and iridescent colonies were observed the suspected colonies were sub-cultured onto selective and non-selective agar to ensure that possible contaminants, the colonies of *Pasteurella* from each plate were collected for presumptive identification according to their morphological characteristics and biochemical tests.

Isolated bacteria were identified according to Barrow and Feltham (1993) using the following tests: indole production test, Catalase test, growth onto Simmons citrate agar medium, and ornithine decarboxylase were studied.

2.2.3 Gram staining revealed small Gram negative coccobacilli arranged singly or in pairs.

2.2.4 Biochemical tests

Positive reactions to indole and ornithine decarboxylase have been described as the most useful biochemical indicators in the identification of *P. multocida*. based on fermentation patterns of dulcitol and sorbitol categorized those positive for sorbitol but negative for dulcitol.

2.3 Isolation and identification of *E coli*

2.3.1 Isolation

Approximately 1 ml fecal sample (homogenized) was suspended into 9 ml of modified Tryptone Soya Broth. Samples were vortexed and incubated overnight at 41°C. After selective enrichment, 50 µl of product was streaked onto Mac Conkey agar for primary isolation of *E. coli* and incubated aerobically at 37°C for 24 hours. The plates were observed for the growth of *E. coli* (pink colony; lactose fermenter). A single, isolated colony was picked and subcultured on Eosin Methylene Blue (EMB) agar for formation of metallic sheen.

2.3.2 Identification

Detection of *E. coli* was carried on colonies that were isolated from fecal swabs samples, single colony with similar characteristics was stained with gram stain. The isolate was examined using bright-field microscopy. KOH test is then employed to confirm the gram reaction. Also, indole, urease, and catalase tests were done.

2.4 Isolation and identification of *Salmonellae sp.*

2.4.1 Isolation

The samples were taken in nutrient broth and SSA (Salmonella Shigella Agar) plates. These were then incubated at 37°C for 24 hours in a bacteriological incubator. After 24 hours the incubated media were then examined for the growth of bacteria. Colorless or translucent colonies and sometimes black color colonies were observed on SS agar. The colony was then subjected to Gram's Method of staining and examined under the microscope for Gram-negative rods. The organisms from the agar media were subcultured into SSA, MCA (MacConkey Agar, EMBA (Eosin Methylin Blue Agar), In the case of SSA, a colorless, translucent, and black colony was observed. In the case of MCA, a colorless and translucent colony was observed. In the case of EMBA, a pale colony without metallic sheen was observed. Thus, a single pure colony was obtained. These pure isolates obtained in this way were used for further study. The Salmonellae colonies were characterized morphologically using Gram's stain. The motility test was performed to differentiate motile bacteria from non-motile one

2.4.2 Identification Differentiation of isolated *Salmonella* spp. using biochemical tests. For this study, isolated organisms with supporting growth characteristics of *Salmonella spp*. were subjected to a sugar (Carbohydrate) fermentation test, TSIA slant reaction, MR (Methyl red) reaction, VP (Voges-Proskauer) reaction, indole reaction, and citrate utilization reaction

3. Special Diagnostic Methods
3.1. Method techniques for detection of *Clostridium perfringens* toxin type A (plc), A (plc + β2), D (plc + etx), and D (plc + β2 + etx) isolated from cattle and buffalo

3.1.1.1Preparation of solutions The solutions are to be prepared extemporaneously. The washing solution must be diluted 20-fold in distilled/demineralized water. The cold solution crystallizes spontaneously. Bring the vial to 21,3 C to make sure that all crystals have disappeared; mix the solution well and withdraw the necessary volume., the dilution solution must be diluted 5-fold in distilled/demineralized water. The conjugates are ready to use the stop solution is ready to use the TMB solution is ready to use it must be perfectly colorless.

Table (A) Composition of the kits

Provided material	BIO K 270/2
Microplate	2
Washing solution (20X)	1 x 100 ml
Coloured dilution solution (5X)	1 x 50 Ml
Theta-toxin conjugate (1X _ Red)	1 x 6 mL
Beta-toxin conjugate (1X_Yellow)	1 x 6 mL
Epsilon- toxin conjugate (1X_blue)	1 x 6 mL
Clostridium perfringens conjugate (1X_ Green)	1 x 6 mL
Positive control (1X)	1 x 4 mL
Single component TMP (1X)	1 x 25 mL
Stop solution (1X)	1 x 15 mL

Table (B) Quality control BIO K 270/2 Multiscreen Ag ELISA enterotoxemia

Validation	Status
Theat > 1.054	Positive > 8.00%
Beta > 1.221	Positive > 7.00%
Epsilon > 1.030	Positive > 8.00%
C perf > 0.993	Positive > 8.00%

3.1.2 Test principle
Specific monoclonal and polyclonal antibodies produced against Alph, Beta, and Epsilon toxins of *Clostridium perfringens* and a monoclonal antibody specific for a structural protein of this bacterium have been producing on alternate rows of 8 x 12 well, microtiter plates. These antibodies allow specifically the capsule of the corresponding toxins or bacteria may be present in the samples. Rows A, C, E, and G have been sensitized with these antibodies, and rows B, C, F, and H are coaled with specific antibodies as control. All samples are diluted in dilution buffer and incubated on the micropipette for 1 hour at 22C + / - 3 C. After the first incubation step, the plate is washed, then conjugates peroxidase, labeled, and anti-pathogen monoclonal or polyclonal antibodies are added to the wells. The plate is then re-incubated for 1 hour at 22C + / - 3 C. After this second incubation step, the plate is washed again, and the chromogen (tetramethylbenzidine TMB) is added. This chromogen has the advantages of being more sensitive than the other peroxidase

chromogen and not being carcinogenic. If specific toxins are presented in the tested samples conjugated remain bound to the corresponding microwell**.** The enzyme catalyzes the transformation of the colorless chromogen into a pigmented compound. The intensity of the resulting blue color is proportionate to the specific pathogen titter in the sample. The enzymatic reaction can be stopped by acidification and the resulting optical density at 450 nm can be read by using a photometer. The signal read for the negative control microwells are subtracted from the corresponding positive microwells. Antigen control is provided with the kits to validate the test results.

3.2 Polymerase chain reaction of *Pasteurella. Multocida*

3.2.1 DNA extraction.

DNA extraction from samples was performed using the QIA amp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56°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 provided in the kit.

3.2.2 Oligonucleotide Primer.

Primers used were supplied from Metabion (Germany) are listed in table (C).

3.2.3 PCR amplification:

Uniplex PCR. Primers were utilized in a 25-µl reaction containing 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 *p*mol concentration, 5.5 µl of water, and 5 µl of DNA template. The reaction was performed in an Applied biosystem 2720 thermal cycler.

3.2.4 Analysis of the PCR Products

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the uniplex products and 30 µl of the multiplex products were loaded in each gel slot. Gelpilot 100 bp plus Ladder (Qiagen, Germany, GmbH) and gene ruler 100 bp ladder (Fermentas, thermo, Germany) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Table le 1: Primers sequences, target genes, amplicon sizes, and cycling conditions.

Target gene	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
			Secondary denaturation	Annealing	Extension		
Kmt1	**460 bp**	**94˚C 5 min.**	**94˚C 30 sec.**	**55˚C 40 sec.**	**72˚C 45 sec.**	**72˚C 10 min.**	**Oie, 2012**
BlaROB1	**685 bp**	**94˚C 5 min.**	**94˚C 30 sec.**	**60˚C 40 sec.**	**72˚C 45 sec.**	**72˚C 10 min.**	
tetH	**1076 bp**	**94˚C 5 min.**	**94˚C 30 sec.**	**60˚C 40 sec.**	**72˚C 1 min.**	**72˚C 10 min.**	**Klima *et al.,* 2014**

Target gene	Primers sequence
	GCTGTAAACGAACTCGCCAC
BlaROB1	**AATAACCCTTGCCCCAATTC**
	TCGCTTATCAGGTGTGCTTG
tetH	**ATACTGCTGATCACCGT**
	TCCCAATAAGCGACGCT

3.3 Serological identification:
The isolated *E. coli* strains were sent for clinical microbiology units in Banha University Faculty of Veterinary Medicine-Food Analysis Center for serological identification

3.3.1 Serological Identification of *E. coli*
The isolates were serologically identified according to Kok *et al.* (1996) by using rapid diagnostic antisera sets (DENKA SEIKEN Co., Japan) for detection of the Enteropathogenic types.

4. Antimicrobial susceptibility: All isolated bacteria were tested for antimicrobial sensitivity using the conventional disc technique according to CLSI(2023) test results.19 different chemotherapeutic agents (BioMerieux discs) were used. The discs used for the in vitro assay namely, Ofloxacin, Doxycycline, Gentamycin, Streptomycin, Ceftriaxone, Erythromycin, Tetracycline, Oxytetracyclines, Lincomycin, Norfloxacin, Chloramphenicol, Neomycin, Ciprofloxacin, Penicillin, Clindamycin, Colistin, Sulphate, Pefloxacin and Aztreonam.

RESULTS

Bacterial examination of the samples affected animals (first group) showed that from 60 examined cattle (30) and buffalo (30) revealed that 50 (83.3%) cases were positive for bacterial infections the number of cattle affected is more than buffalo. The percentage of infected cattle was 50% from all positive cases while that of buffalo. 33.3% from positive cases which constitute (83.3%).

On the other hand, the bacteriological examinations of dead animals (second group) revealed that 30 (100%) cases were positive for bacterial infections the number of dead cattle is the same number of dead buffalo, and the percentage of dead animals is 100% from all positive cases. The mortality rate was higher in newly born calves than in old ages as shown in Table (1).

Table 1: Influences susceptibility to bacterial infection in two groups.

Total number		**first group**		**Second group**	
100		60		40	
Cattle	Buffalo	Cattle	Buffalo	Cattle	Buffalo
		Positive	results		
50	50	30	20	20	20
(50%)	(50%)	(50 %)	(33. 3%)	(50%)	(50%)
(100%)		(83.3%)		(100%)	

The bacteriological findings:
Identification of isolates from all samples resulted in the detection of 40 bacterial isolates. The isolates constituted 4 genera from different species of both Gram-positive and Gram-negative bacteria. Also, aerobic and anaerobic micro-organisms. The following strains were recognized as the isolated ones: Out of the 100 samples (first and second groups) that were tested, these isolates were found in 90 samples with positive results (about 90%) *Clostridium perfringens* 24 (46.7, 33.3), *Pasteurella multocida* 17 (33.3%, 23.3), *Escherichia coli* 6 (13.3, 6.7 %), and *Salmonella sp*. 3 (6.7, 3.3%) in cattle and buffalo respectively as shown in table (2).

Table 2: Incidence of the isolated micro-organisms from Cattle and Buffalo of First groups.

Micro organisms	Positive samples			
	Cattle 30/30 (100%)		Buffalo 20/30(66.7%)	
	NO	%	NO.	%
Clostridium perfringens	14	46.7	10	33.3
Pasteurella multocida	10	33.3	7	23.3
Escherichia coli	4	13.3	2	6.7
Salmonella sp.	2	6.7	1	3.3
Total	30	100	15	66.7%

Whereas, isolates from the Second group (immediate death) *Clostridium perfringens* 18 (50, 40), *Pasteurella multocida* 11 (30%, 25), *Escherichia coli* 8 (15, 25 %), and *Salmonella spp.* 3 (5, 10%) in cattle and buffalo respectively as shown in table (3).

Table 3: Incidence of the isolated micro-organisms from Cattle and Buffalo of Second groups.

Microorganisms	**Positive samples**			
	Cattle 20/20 (100%)		Buffalo 20/20 (100%)	
	NO	%	NO.	%
Clostridium perfringens	10	50	8	40
Pasteurella multocida	6	30	5	25
Escherichia coli	3	15	5	25
Salmonella sp.	1	5	2	10
Total	20	100	40	66.7%

Culture sensitivity test C L S I. Performance Standards **(2023):**
Antibiogram of bacteria isolated from diseased and immediately dead animals have been shown in tables (4) from all the 19 antimicrobial agents used for *Clostridium perfringens* isolates were sensitive for (Ofloxacin – oxytetracycline – Aztreonam). For *Pasteurella. Multocida* isolates (Gentamycin, Ceftriaxone, and Norfloxacin) were sensitive (100 %), Chloramphenicol, Neomycin and Ciprofloxacin were sensitive

(94 %, 84 %, and 74 %, respectively) whereas Penicillin, Streptomycin, Clindamycin, Colistin Sulphate, Pefloxacin and Aztreonam all of them were resistant (100 %). Erythromycin and Tetracycline were resistant (94 %), Lincomycin was resistant (80%) and Doxycycline was resistant (76%). Antimicrobial sensitivity test revealed that gentamycin (30µg) was the most effective antibiotic against most isolated microorganisms of *E. coli.* However, oxytetracycline (30µg) were resistance (78%). Antimicrobial sensitivity test revealed that ciprofloxacin and ceftriaxone were the most effective antibiotic against *salmonalla sp.*. However, Streptomycin were resistance (82%) as shown in table (4).

Table 4: Antibiogram test of the isolated strains.

Antimicrobial agents	***Pasteurella. Multocida***		***Clostridium. perfringens***		***E. coli***		***Salmonella* sp.**	
	S%	**R%**	**S%**	**R%**	**S%**	**R%**	**S%**	**R%**
Garamycin (30µg) G	0	0	0	0	100	0	0	85
Ofloxacin((5µg) OFX	0	0	100	0	0	78	0	0
Doxycycline(30µg) DO	0>	76	0	0	0	0	0	0
Gentamycin(10µg) GEN	>100	0	0	100	0	0	0	0
Ceftriaxone(30µg) CRO	0	0	0	0	0	0	100	0
Streptomycin(10µg) ST	0	100	0	0	0	0	0	82
Ceftriaxone(30µg) CRO	>100	0	0	100	0	0	0	0
Erythromycin(15µg) ERY	0>	94	0	0	0	0	0	0
Tetracycline(30µg) TE	0	94	0	0	0	0	0	0
Oxytetracycline(30µg) T	0	0	100	0	0	100	0	0
Lincomycin(40µg) L	>0	80	0	0	0	0	0	0
Norfloxacin(10µg) NF	100	0	0	100	0	0	0	0
Chloramphenicol (30µg) C	94	0	0	0	0	0	0	0
Neomycin(30µg) N	84	0	0	0	00	0	0	0
Ciprofloxacin(5µg) CIP	74	0	0	0	0	0	100	0
Penicillin (6µg) P	0	100	0	0	0	0	0	0
Clindamycin(15µg)	0	100	0	0	0	0	0	0
Colistin(40µg) CT	0	100	0	0	0	0	0	0
Sulphate	0	100	0	0	0	0	0	0
Pefloxacin(10µg) PO	0	100	0	0	0	0	0	0
Aztreonam(30µg) AZ	0	100	100	0	0	0	0	0

S: sensitive %: percent of sensitivity
R resist % R% percent of resistance

Table 5: Frequency of *Clostridium perfringens* toxin types isolated from cattle and buffalo in sampled areas of Assiut Governate

Code	C	484	981	988	989	5136						
	1	2	3	4	5	6	7	8	9	10	11	12
A	1.958	1.847	1.933	1.699	1.847	1.485						
B	0.098	0.097	0.109	0.094	0.088	0.056						
C	1.995	0.095	0.117	0.162	0.088	0.077						
D	0.096	0.094	0.115	0.125	0.079	0.071						
E	1.805	0.098	0.094	0.153	0.084	0.074						
F	0.099	0.089	0.127	0.113	0.082	0.072						
G	2.046	1.925	1.748	1.854	2.005	1.899						
H	0.103	0.056	0.112	0.097	0.117	0.079						
ϴ	1.860	1.750	1.824	1.605	1.759	1.429	0.000	0.000	0.000	0.000	0.000	0.000
β	1.899	0.001	0.002	0.037	0.009	0.006	0.000	0.000	0.000	0.000	0.000	0.000
ε	1.706	0.009	-0.033	0.040	0.002	0.002	0.000	0.000	0.000	0.000	0.000	0.000
C.p	1.943	1.869	1.636	1.757	1.888	1.820	0.000	0.000	0.000	0.000	0.000	0.000
ϴ	100	94	98	86	95	77	0	0	0	0	0	0
β	100	0	0	2	0	0	0	0	0	0	0	0
ε	100	1	-2	2	0	0	0	0	0	0	0	0
C.p	100	96	84	90	97	94	0	0	0	0	0	0

Result: C = Positive Control Antigen **Sample no. 484,981,988,989,5136 = *C. perfringens* Type A**

Lot No: ENT22L22

Analyst Sign and date **T.M. Sign and date**

Isolation of Toxin types Isolated from *Clostridium perfringens* in Buffalo and Cattle. After the isolation of *Clostridium perfringens* and identification five strains of *Clostridium perfringens* were examined for identification of toxin genotypes in both buffalo and cattle, was recorded that type A (plc) showed maximum incidence in both cattle and buffalo, as all the examined animals were found prone to this genotype (Table 5).

Polymerase chain reaction:

After the isolation of *P. multocida* and identification ten strains of *P. multocida* were examined for identification *P. multocida* in Animal Health Research Institute the results were observed (table 6).

Table 6: PCR analysis of *Pasteurella. multocida* isolates from cattle and buffalo in sampled areas of Assiut Governate.

Sample	*P. multocida Kmt1 gene*	*BlaROB1 gene*	*tetH gene*
1	+	+	+
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	-	ND	ND
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+

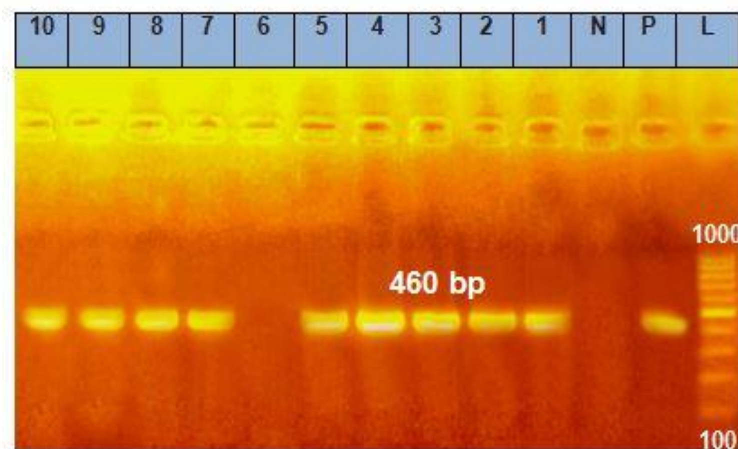

Fig (1): PCR analysis of *Pasteurella. multocida* isolates showing 460 bp lanes for Kmt1 gene. Lane L: 100 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lane 1,2,3,4,5,7,8,9,10: Positive samples, Lane 6: Negative sampl**e.**

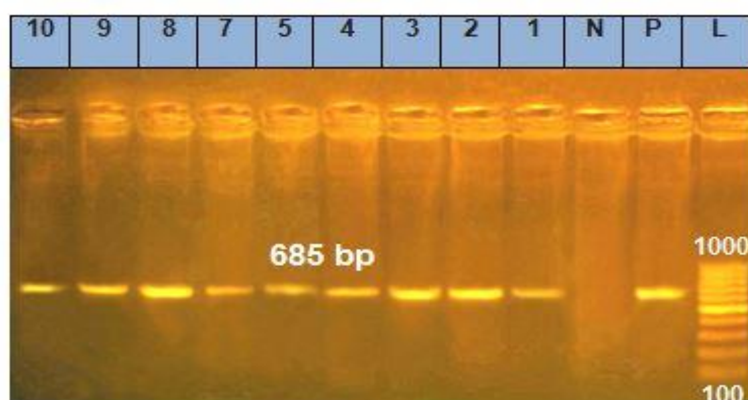

Fig (2): PCR analysis of *Pasteurella. multocida* isolates showing 685 bp lanes for BlaROB1 gene. Lane L: 100 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lane 1,2,3,4,5,7,8,9,10: Positive samples.

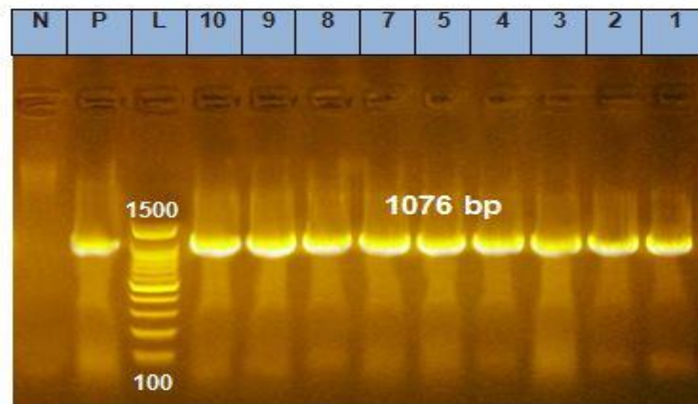

Fig (3): PCR analysis of *Pasteurella multocida* isolates from ????? showing 460 bp lanes for the *tetH* gene. Lane L: 100 bp DNA ladder, Lane P: Positive control, Lane N: Negative control, Lane 1,2,3,4,5,7,8,9,10: Positive samples

6-Serological identification of *E. coli*

After the isolation of *E. coli* and identification 7 strains of *E. coli* were examined for Serological identification *E. coli* in Animal Health Research Institute the results were observed in (table 7).

Table 7: Serological identification of *E. coli* strain from first and second groups.

Identified strains	No of total isolates	Cattle	Buffalo
E. coli O104: H4 EPEC	2 isolates	1 isolate	1 isolate
E. coli O126 EPEC	1 isolate	3 isolates	----
E. coli O111: H2 EPEC	1 isolate	1 isolate	-----
E. coli O113: H21 EPEC	1 isolate	=====	1 isolate
E. coli O91 EPEC	1 isolate	1 isolate -	====
E. coli O103: H2 EPEC	1 isolate	==	1 isolate
E. coli O26: H11 EPEC	1 isolate	===	1 isolate

DISCUSSION

Data of the cattle and buffalo mortalities due to some bacteriological agents such as *Clostridium perfringens, Pasteurella multocida, Escherichia coli,* and *Salmonella* sp. were collected and subjected to statistical analysis. It was found that the overall mortality for cattle was higher than for buffaloes (50.0 and 33. 3%) respectively. In the examined areas, deaths were mainly confined to young animals more than older ages. Outbreaks of Hemorrhagic septicemia (*Pasteurella multocida*) occurred deaths were scattered over all age groups. In buffalo species mortality is considered one of the major causes of fertility loss, especially in animals that are not mated during their reproductive period Daneshmand *et al.* (2022).

The results indicated that 100% of all samples taken were positive bacteriologically for the first (diseased) and second (immediately dead) groups in cattle, whereas results recorded (20/30 – 6.67%) for the first (diseased) and (30/30 – 100%) for (immediately death) second group. The most common isolates observed were *Clostridium perfringens* (46.7%), *Pasteurella multocida,*

(33.3%) *E. coli* (13.3%), and *Salmonella* sp. (6.7%) of cattle first(diseased) group, whereas results revealed that (33.3, 23.3, 6.7 and 3.3)of *Clostridium perfringens, Pasteurella multocida, Escherichia coli,* and *Salmonella* sp. respectively isolated from the group of buffalo as shown in (Table 2) The results indicated that bacteriologically examination for the second (immediately death) groups in cattle, (50, 30, 15 and 5 %) of *Clostridium perfringens, Pasteurella multocida, Escherichia coli* and *Salmonella sp* respectively whereas .results recorded (40, 25, 25 and 5 %) in buffalo as shown in (Table 3) *E. coli* also, maybe a common pathogen involved in mortality rates in cattle and buffalo. As Bio Numbers. (2022). Serological characteristics of *E. coli* strains according to pathogenic mechanisms are classified into four major categories enterotoxigenic *E. coli* (ETEC). Enteropathogenic E. coli (EPEC). enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E.coli* (EHEC).), which are represented by different serotypes based on (cell wall lipopolysaccharides), H(flagellar protein) and K (capsular polysaccharide or envelope)antigens (Lehman and Segal (2020). In our study, 8 random E. coli serotypes were recorded to O 104, O126, O111, O113, O91, O103 and O26.

The serogroup O78 which was isolated from hemorrhagic outbreak in cattle and buffalo in some areas O was not isolated in this study. Moreover, the serotype (O22, O55, O10) which was not isolated in this study may be attributed to the different locality of each study. *Streptococcus* spp. and *Salmonella* sp. are also, opportunists & have been associated with sudden death as recorded in cattle and buffalo (Lehman and Segal (2020). and was considered the least agent affection in cattle and buffalo as recorded also by Selim *et al.,* (2017).

Antibiogram of bacteria isolated from diseased and immediately dead animals have been shown in tables (4) From all the 21 antimicrobial agents used for *Clostridium perfringens* isolates were sensitive to (Ofloxacin – Oxytetracycline – Aztreonam) and were resist for Gentamycin, Ceftriaxone and Norfloxacin (100 %). For *Pasteurella. multocida* isolates were sensitive to (Gentamycin, Ceftriaxone, and Norfloxacin) (100 %), and sensitive to Chloramphenicol, Neomycin, and Ciprofloxacin (94 %, 84 %, and 78 %, respectively) whereas resisting for Penicillin, Streptomycin, Clindamycin, Colistin Sulphate, Pefloxacin, and Aztreonam (100 %). And resist Erythromycin and Tetracycline (94 %), Lincomycin (80%), and Doxycycline (76%). Antimicrobial sensitivity test revealed that Gentamycin (30µg) was the most effective antibiotic against most isolated microorganisms of *clostridium perfringens* whereas oxytetracycline (30µg) for *E. coli* while ciprofloxacin and ceftriaxone were the most effective antibiotic against *salmonella sp.*. However, Garamycin and Streptomycin were resistant (85 and 82%) respectively. (30µg).

Clostridium perfringens is only an anaerobic bacterium that causes high mortality in both cattle and buffalo. The main cause of death by *Clostridium perfringens* was toxin type A disagreed with [Lehman and Segal (2020)] *Pasteurella multocida* is a species of aerobic Gram-positive bacterium that is considered a significant bacterium that causes high mortality in both cattle and buffalo. *Escherichia coli* is the second-most significant microbe that causes death in both cattle and buffalo, from 6 and 8 *Escherichia coli* strains isolated from the first and second groups respectively, 8 serotypes (O104, O126, O111, O113, O91, O103, and 26) identified from cattle samples, whereas (O104, O113, O103, and O26 were identified from buffalo samples that disagreed with (Ashgan *et al.* 2013)

The result of PCR for amplification of Kmt1 mBlaROB and1etH genes in *Pasteurella multocida* showed that Kmt1 mBlaROB and1etH genes were amplified in all tested

isolates, which disagreed with (Yeruham, *et al.,*2016)

CONCLUSION

From this study, we concluded that *Clostridium perfringens, Pasteurella multocida Escherichia coli* and *Salmonella sp*. are the most common pathogens which cause sudden death in cattle and buffalo. Recommendation for treating and controlling the clinical cases of diseased animals with the drug of choice based on the isolated causative organism and the antibiogram study which is Ofloxacin – oxytetracycline – Aztreonam (*Clostridium perfringens*) Gentamycin, Ceftriaxone and Norfloxacin (*Pasteurella multocida*) garamycin and oxytetracycline *(Escherichia coli*) and ciprofloxacin and ceftriaxone were the most effective antibiotic against *salmonella sp*. *Clostridium perfringens* were examined for identification of toxin genotypes in both buffalo and cattle it was recorded that, type A(plc)in PCR result assured that strains are *Pasteurella multocida* and have Kmt1 mBlaROB and1etH genes virulence genes .

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بعض المسببات البكتيريه المسئوله عن الامراض والنفوق في الماشيه والجاموس

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تشكل حوادث الوفيات الجماعية في الماشية والجاموس مصدر قلق متزايد. في ظل ظروف التغير العالمي السريع، فإن الاستجابات الانتهازية في التعايش البكتيري، نتيجة للضغوطات البيئية التي تؤدي إلى تنشيط بعض العوامل البكتيرية التي قد تكون متورطة بشكل متزايد في حالات الموت. في هذا البحث تحدثنا عن بعض العوامل البكتيرية المسببة للموت المفاجئ في الأبقار والجاموس. تم جمع عينات من إفرازاتالأنف و البراز للفحص البكتريولوجي من (٦٠ بقرة وجاموس مريضة ظاهريا ٣٠ من كل نوع مختلف الأعمار والجنس والتي يشتبه سريريا في إصابتها بأعراض تنفسية وإسهال (المجموعة الأولى) و ٤٠ محتويات الكرش والكبد والطحال و الرئة كعينات نسيجية من الحيوانات المذبوحة من الأبقار والجاموس ٢٠ من كل *نوع (المجموعة الثانية).تم جمع عينات من عدة مواقع بمحافظة أسيوط وإرسالها للمعمل.أشارت نتائج المجموعة الأولى إلى أن ٨٣,٥% (٥٠) ٣٠ من الأبقار والجاموس ٢٠ جاموس كانت إيجابية للعدوى البكتيرية ولكن ١٠ منها أظهرت نتائج سلبية للعدوى البكتيرية في الجاموس، في حين أشارت المجموعة الثانية إلى ١٠٠% (٤٠) (٢٠ أبقار و ٢٠ جاموسة). أظهرت الفحوصات البكتريولوجية للمجموعة الأولى أن الميكروبات المعزولة هي: كلوستريديوم بيريفريندز (٤٦,٧%)، باستوريلا ملتوسيدا (٣٣,٣%)، اشريشيا كولاي ا (١٣,٣%) سالمونالا((٦,٧%) في الأبقار ولكن في الجاموس سجلت كلوستريديوم بيريفريندز (٣٣,٣%) (٢٣,٣%)، اشريشيا كولاي (٦,٧%)، سالمونالا سب (٣,٣%)، من جهة أخرى أظهرت التحريات البكتريولوجية للمجموعة الثانية أن الكائنات الحية المعزولة هي: كلوستريديوم)(٥٠%)، باستوريلا ملتوسيدا (٣٠%)، اشريشيا كولاي ا (١٥%) سالمونالا سب. (٥%) في الماشية بينما في الجاموس أظهرت كلوستريديوم بيريفريندز (٤٠%) ، باستوريلا ملتوسيد(٢٥%) ، اشريشيا كولاي ا (٢٥%) سالمونالا(١٠%)، إجمالي ٤٢ عزلة من بكتيريا كلوستريديوم بيريفريندز معزولة من المجموعة الأولى والثانية، ١٢ عزلة تم فحصها لمعرفة نوع السم باستخدام Multiscreen Ag ELISA Enterotoxemia وكان النمط السمي الأكثر حدوثاً هو النوع A (الجاموس: ٥٠/٥؛ الماشية: ٥٠/٧) كانت إجمالي ١٤ عزلة من بكتيريا E coli من العينات المفحوصة من المجموعة الأولى والثانية إيجابية مصليا لـ O111 وO104 وO26 وO113 وO91 وO103 وO126. أظهرت نتائج تفاعل البلمرة المتسلسل أن عشر سلالات من ٢٨ عزلة من المجموعة الأولى والثانية كانت من عزلات باستوريلا ملتوسيد وبعضها يحمل جينات Kmt1 وBlaROB1 وtetH.

تم تطبيق المضادات الحيوية على مسببات الأمراض البكتيرية المعزولة ووجد أن أوفلوكساسين - أوكسي تتراسيكلين - أزتريونام (كلوستريديوم بيريفريندس) جنتاميسين، سيفترياكسون ونورفلوكساسين (باستوريلا مولتوسيدا)، جاراميسين وأوكسيتتراسيكلين (إيشيريشيا كولاي) وسيبروفلوكساسين وسيفترياكسون هي المضادات الحيوية الأكثر فعالية ضد السالمونيلا sp. كان الدواء المفضل لعلاج الحيوانات المصابة