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The incidence of salmonellae isolated from diarrheic cattle and sheep with special reference to the virulence of the most prevalent *Salmonella*

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Abstract

Incidence of *Salmonella* was determined through examination of 180 diarrheic faecal samples from calves, adult cows, lambs, and adult sheep10 %, 6.6 %, 11.6 % and 6.6 % respectively. Serotyping of the isolates revealed 4 serovars including *S.* Dublin, *S.* Virchow, *S.* Cerro and *S*. Typhimurium which encountered the most frequent serovars (50%).

All isolates of *S.* Typhimurium were positive to Congo red in different intensities in the dye uptake. MSHA strains were higher than MRHA strains in all *S.* Typhimurium when used bovine and sheep RBCs while MRHA strains higher when used RBCs of G. pig &chicken. Isolated from adult cow and sheep show no haemagglutination with RBCs of chicken, while, isolates from adult cow show no haemagglutination with RBCs of G.pig. Eight isolates of *S.* Typhimurium had ability to survive in serum after 1,3and 6 hours and could grow in serum. All isolates had ability to adhere and invade HEP-2 cell (causing death in cells with different degree) except one isolate from adult sheep could not invade HEP-2 cell. Mortality rate in mice were 53.3%, 40 %, 66.7 % and 60 % of isolated *S*. Typhimurium from calves, adult cows, lambs and adult sheep respectively. On the other hands, the entrotoxigenicity were 66.7% and 0 %in *S*. Typhimurium isolated from calves and adult cows while were 66.7and 0 % in *Salmonella* isolated from lambs and adult sheep respectively.

It was found that all tested isolates were completely resistant to colistin sulphate and lincomycin. On the other hand, gentamicin, flumequine and norfloxacin gave good susceptibility followed by ofloxacin.

Key words: *S. Cerro, S. Typhimurium, gentamicin, flumequine*

Introduction

Cattle and sheep are the important animals in Egypt for, meat, milk and wool productions.

Diarrhea is a problem that may lead to great economical losses, as it consider the most important cause of calf morbidity and mortality especially among newly born buffalo and cow calves in Egypt and all over the world **(Bayomi *et al*., 1996)**, and most common cause implicated in out breaks of food born infection around the world **(Abou –Zeed *et al*., 2000)**

Salmonellae are among the most common bacteria that cause diseases to animals and man, it

produced low production and great economic losses in animals specially *S.* abortus ovis which leads to abortion in sheep. Also, salmonellae cause enteritis, metritis and septicemia; all were potentially lethal to both ewes and lambs **(Moredun foundation, 2007)**. Also in calves results in marked to serve dehydration, depression, weakness, partial loss of appetite and some cases suffered from recumbency. **(Moustafa *et al*., 2007).** The infection of Salmonella in calves appears in the form of septicemia and diarrhea and in pregnant cows many abortion were occurred **(Santos *et al.,* 2002).** Calves suffering from acute clinical salmonellosis may shed10^{8}-10^{10} *Salmonella* /gram feces.

Salmonellae constitute a hazard to public health as all serovares can produce diseases to human **(WHO, 2006)**. It causes typhoid, paratyphoid, septicemia and enteritis to man. The extensive use of antibiotics, not only in human and veterinary medicine, but also in livestock production for disease prevention or as growth-promoting feed additives, has led to a serious increase in, and spread of, multiple antibiotic-resistant bacteria **(Moellering, 1998)**.

The current study was designated to shed some light on the following; isolation and identification of salmonellae from faecal samples of diarrheic calves, cow, lamb and sheep. Serological identification of isolated salmonellae. Studying the virulence of the most prevalent *Salmonella* including Congo red (C.R) binding activity, haemagglutination test (HA), serum resistance, pathogenicity in HEP-2 cells &mice, detection of heat stable enterotoxin and antibiogram.

Material and Methods

Samples collection.

One hundred and eighty faecal samples were collected from diarrheic grazing cattle and sheep in farms or sporadically (adults, calves and lambs) from rectum with sterile gloves located in Kafr El-Sheikh and Giza. Each sample was collected in sterile polyethylene plastic bag and send to laboratory as soon as possible in ice box.

Isolation of salmonellae.

Faecal samples were directly cultured onto XLD agar and Brilliant green agar. The plates were incubated at 37^{o} C for 24 hours, or indirect culturing by putting 10 grams of each sample in 100 ml of buffered peptone water as pre-enrichment and incubated at 37°C for 20 hours. Then 0.1 ml of pre-enriched broth was transferred to 10 ml of Rappaport Vassiliadis selective broth then incubated at 41.5°C for 24 hours. Also another 1 ml of pre-enriched broth was transferred to 10 ml of tetrathionate broth and incubated at 37^{o}Cfor 24 hours and streaked onto XLD agar and Brilliant green, the plates were incubated at 37^{o} C for 24 hours.

Identification of salmonellae.

Suspected colonies were described for their morphological and characteristic appearance. Pure cultures of the isolates were identified biochemically according to **Quinn *et al.* (2002).**

Final results were confirmed by using the API 20E, biochemical identification kit (bioMe´rieux, Marcy l'Etoile, France.)

Serological identification. of suspected *Salmonella* isolates were carried out using agglutination technique according to the Kauffmann - White scheme as described by **Kauffmann (1973)**. The typing antisera were obtained from Denka Seiken Co. Ltd, Tokyo, Japan.

Virulence factors of *S.* Typhimurium.

Congo red (C.R) binding activity. according to **Berkhoff and Vinal (1986).** Isolates were tested for their growth status onto Congo red medium and the reaction was best seen after 24 hours of incubation at 37ºC and then left at room temperature for additional 2 days Congo red (CR +) serovars was indicated by the development of bright or orange red colonies. Different intensities in the dye uptake were expressed as (+) and (++) whereas Congo red negative (CR^{-}) organisms showed white colonies (did not bind the dye).

Haemagglutination test (HA) according to **Evans *et al*. (1979).**

Different blood samples were collected from various origins (bovine, sheep, guinea pig and chicken). One ml of 3.8 % citric acid in dis-

tilled water was added to 9 ml of the blood. Blood was diluted 1:4 with phosphate buffered saline (PBS) pH 7.2 to test for HA and 1:4 with 1% D-mannose in PBS to test for mannose resistance haemagglutination (MRHA). Bacterial cells from each isolate were obtained from cultures grown for 18 hours at 37°C on colonization factor agar (CFA) plates. Growth was picked up and mixed with a drop of the appropriate species of blood on microscopic slide at room temperature and observed from 1-2 minutes. Haemagglutination was observed by the naked eye. HA was recorded as M.R. if occurred with and without D-mannose and as M.S. if HA was inhibited in the presence of D-mannose.

Serum resistance (Barrow and Hill, 1989).

Blood was obtained from apparently healthy cows and sheep (serum of cow used in serum resistance of calves and adult cow, serum of sheep used in serum resistance of lambs and adult sheep) and allowed to clot for 2 hours at room temperature. The serum was collected, pooled and filtered through membrane filter 0.45 µm. Nutrient broth culture of each isolate was incubated at 37ºC for 24 hours and centrifuged at 1500 x g for 15 minutes. The pellet was resuspended in an equal volume of phosphate buffered saline (pH 7.4) to obtain about $2X10^6$C.F.U. / ml. Two tests were carried out the first to detect the survival of isolates and the second to observe their growth in serum.

Survival in serum.

Twenty five µ1 of bacterial suspension were inoculated in a well of haemagglutination tray containing 75µ1 of serum. The inoculated serum was incubated at 37°C and after 0, 1, 2, and 3 and 6 hours, 20 µl was plated into tryptose soya agar medium. After incubation the number of colonies was counted. Isolates of examined strains were classified as serum resistance when the number of colonies in 1, 3 and 6 hours samples was greater than that in 0 hour samples.

Growth in serum. using a modified method described by **Moll *et al*. (1979)**. Two wells were used for each isolate. 30µ1 of 2.5% glucose containing 0.25% bromothymol blue were pipetted into two wells of haemagglutination tray and then dried over night. 120µ1of serum was added to one well and to another well 1% pepton water (pH7.4) was added. Both wells were inoculated with the examined strain; the tray was covered with polyethylene bag and incubated for18 hours at 37ºC. Development of yellow colour with bacterial pellet in bottom of well indicated bacterial growth. While, blue colour indicated no bacterial growth.

pathogenicity in HEP-2 cells.

Adherence assay by (Donnenberg and Natars, 1995).

10 µl of overnight bacterial cultures in peptone water (containing 1% D mannose) were inoculated into cover slip-containing 24 well plates, which had been seeded with 5 x 10^5 HEP-2 cells 48 hours-age. Cultures were incubated at 37 °C for 3 hours. The cells were washed. Fresh Hanks 199 was added and then the cells were incubated for further 3 hours. Then cells were fixed with 3% formalin and stained with Geimsa solution. The adhesion was determined by light microscopy covering the whole slide. Bacteria were recorded as adhesive if a cluster of at least 10 bacteria adhered per HEP-2 cell.

Invasion assays (Tang *et al.*, 1993 and Janda and Abbott, 1998).

10 µl of bacterial culture in peptone water were incubated with HEP-2 cells for 2-3 hr. to allow attachment and penetration of epithelial cells. Gentamicin (which is unable to penetrate mammalian cells) as added to eliminate extracellular bacteria, and the tissues cell sheet was again incubated to allow multiplication of the bacteria that had invaded. The cell sheet was washed 3 times with PBS, fixed and stained by Giemsa. Recorded various degree of cell death.

Pathogenicity in mice (Mouse lethality test) **(Carsiotis *et al*., 1984)**

A*Preparation of microbial suspension.

All different serovars of *Salmonella* were grown in tryptic soy broth (TSB) for overnight at 37°C pelleted at 4.500 xg for 5 minutes at 4 °C then washed 3 times in sterile normal saline solution .The pellet was resuspended in 3 ml sterile normal saline solution to obtain an ap-

proximate bacterial count of $3x10^9$CFU ml. It was adjusted using McFarland opacity standard.

Experimental design.

Forty five Adult albino white mice (male and female) were 6-8 weeks of age at the time of experiment. All mice were examined bacteriologically to ensure their free from pathogens. 5mice were kept separately as a control group and 5 mice were used for each isolate. All of the mice were deprived of water overnight 0.25 ml of $3x10^9$ CFU ml of an overnight broth was orally deposited behind the upper incisors of mice (care was taken not to scrape the mucosa of the mouth of the mice). Mice of each tested strain were kept separately, the mice were observed twice daily up to 21 days for signs of illness or death. An attempt was also made to re isolate the *Salmonella* from different organs (liver and intestine).

Detection of heat stable enterotoxin. (Robins -Brown *et al.*, 1993).

Preparation of culture filtrate via inoculation of each isolate in tryptone soya broth and incubated over night at 37ºC.Then 10 ml of culture was placed in 200 ml of medium containing 2% casamino acid, 1% yeast extract and 0.4% glucose pH (8.5) in 250 ml flask. The inoculated flasks were incubated on a rotatory shaker 200 rpm at 37ºC for 18 hours then centrifuged at 12000 xg for 10 minutes. The supernatant was filtrated through Millipore membrane filter pore 0.45µm and stored at -20ºC till used. A part of sterile medium was used as control. Infant mouse assay 0.1 ml of each filtrate was injected through the abdominal wall into milk filled stomach of each 5 mice 2-4 days old for each examined strain and 3 infant mice were injected by 0.1 ml of sterile medium and were used as negative control. After 4 hours, the mice were killed and the entire intestine was removed. The intestine and remaining body were weight to calculate the ratio of intestine weight/remaining body weight. Ratio greater than (0.083) was recorded as positive test for enterotoxins.

Antibiogram.

This was done using disc diffusion standard technique according to **Finegold and Martin (1982)** and **Quinn *et al.* (2002)** with Mueller Hinton medium, using the following 10 (Oxoid) discs, amoxicillin (25µg), ciprofloxacin (5µg), colistin sulphate (50µg), enrofloxacin (5µg), flumequine (30µg), gentamicin (10µg), lincomycin (10µg), norfloxacin (10µg), ofloxacin (5µg) and tetracycline (30µg). The results were interpretated according to the manual supplied by Oxoid Company **(CLSI, 2009).**

Results and Discussion

This study revealed that both diarrhoeic cows and sheep can harbor *Salmonella* in different incidences and serovars. Incidence of *Salmonella* was determined through the examination of 180 diarrheic faecal samples from calves, adult cows, lambs and adult sheep 10 %, 6.6 %, 11.6 % and 6.6 % respectively. It is clear that the rate of incidence was varying according to species and age. It appears that it is high in sheep than calves and in young's than adults. Serotyping of the isolated salmonellae from examined samples revealed 4 serovars including *S.* Typhimurium, *S.* Dublin, *S.* Virchow and *S.* Cerro. The most frequent serovars encountered were *S.* Typhimurium (50%). S. Cerro isolated only from calves meanwhile, *S.* Virchow isolated only from lambs as reported in Table (2).

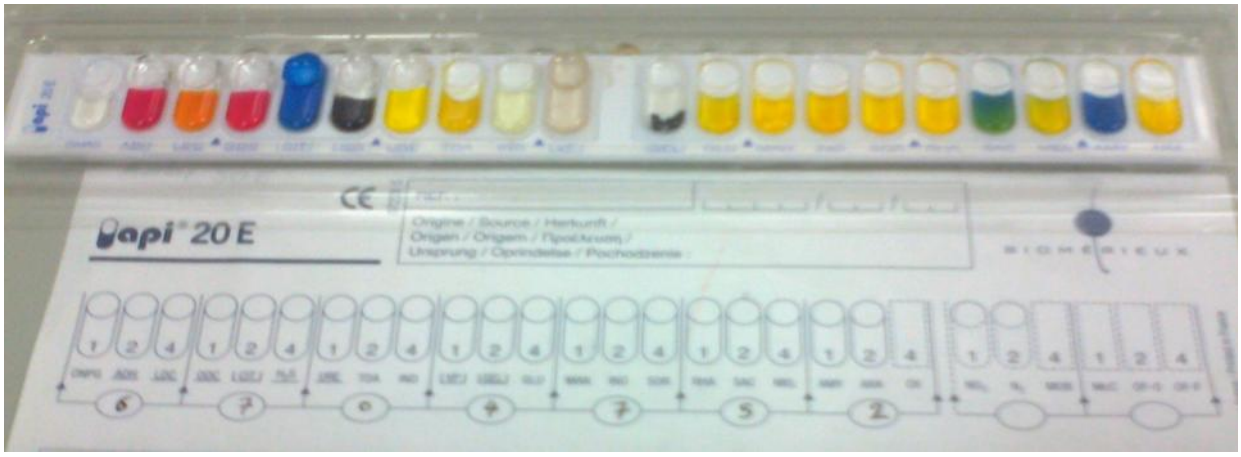

Photo (1). A PI 20 of *Salmonella* Typhimurium isolated from diarrheic cattle and sheep.

Table 1. Incidence of salmonellae in diarrhoeic cows and sheep.

Animals	Age	No. of examined samples	No. of positive	%*
Cow	Calves	60	6	10.0%
	Adult	30	2	6.6%
Sheep	Lambs	60	7	11.6%
	Adult	30	2	6.6%

*The percent was calculated according to the number of sample of each species.

Table 2. Serovars of salmonellae isolated from diarrhoeic cow and sheep.

Animals	Age	NO of *salmonella*	Serovas	No.	%*
Cow	Calves	6	*S*. Typhimurium *S*. Dublin *S*. Cerro	3 1 1 1	50 16.7 16.7 16.7
	Adult	2	*S*. Typhimurium *S*. Dublin	1 1	50 50
Sheep	Lambs	7	*S*. Typhimurium *S*. Dublin *S*.Virchow	3 2 2	42.9 28.6 28.6
	Adult	2	*S*. Typhimurium *S*. Dublin	1 1	50 50

*The percent was calculated according to number of salmonellae of each age and species.

In this concern, **Salman and Tanios (2004)** isolated (10.59 %) *Salmonella* from diarrhoeic calves (4.71%) belonged to *S.*Typhimurium, (2.35 %) belonged to *S*. Dublin and *S*. Enteritidis in each and (1.18 %) was *S*. Meleagrids. Meanwhile**, Moustafa *et al*., (2007)** recorded the incidence of *Salmonella* were (16% and12%) in feacal samples of diarrhoeic cows & buffalo calves their age ranged from birth up to 2 month old and identified as *S.* Typhimurium and *S*. Enteritidis. Also, **Rafequ and Abd-El Aty (2007)** found the incidence of *Salmonella* in calves suffering from diarrhea was (17.5%), four serovars were elucidated namely, *S.*Typhimurium (7.3%), *S*. Dublin (5.1%), *S.* Enteritidis(2.9%) and *S.* Anatum (2.2%). However**, Salman *et al*., (2007)** reported the incidence of *Salmonella* was (11.54%) in diarrheic buffalo calves strains representing four serotypes. *S.* Typhimurium was the most prevalent then *S.* Meleagrdis, *S.* Arizona and *S.* Virchow. Also **Ibrahim and El- Gohary, (2007)** recorded that the incidence of *Salmonella* in diseased lambs and diseased adult sheep were (12.73%) and (6.67%) respectively, the isolated serovar were *S.* Cerro, *S.* Montevideo, *S.* Sendiogo and *S.* Typhimurium. Otherwise, **Ahmed *et al.*, (2009)** isolated nine salmonellae from 220 fecal samples from neonatal calf diarrhea in Egypt and the isolated serovar were *S*. Typhimurium and *S*. Enteritidis. Finely, **Bolton *et al*., (2012)** detected *Salmonella* in bovine faeces on four mixed farms; *Salmonella* serotypes included *S*. Dublin, *S*. Kiel and *S*. Typhimurium.

The obtained data in Table (3) illustrate virulence markers of 8 *S*. Typhimurium isolates including Congo red, haemagglutination , serum resistance**,** pathogenicity in HEP-2 cells& mice and detection of enterotoxin. All isolates were positive to Congo red in different intensities in the dye uptake. Congo red (C.R) binding activity could be used as phenotypic marker to distinguish between invasive and non-invasive isolates **Berkhoff and Vinal, (1986)**.

The results of haemagglutination varied according to used RBCs collected from various origins (bovine, sheep, G.pig & chicken) and presence or absence of D-mannose. MSHA strains were higher than MRHA strains in all *S.* Typhimurium when used bovine and sheep RBCs while MRHA strains higher when used RBCs of G. pig &chicken. *S.* Typhimurium isolated from adult cow and sheep show no haemagglutination with RBCs of chicken meanwhile*, S.* Typhimurium isolated from adult cow show no haemagglutination with RBCs of G.pig. Although there were variation in haemagglutination among *S.* Typhimurium from calves, adult cow, lamb and adult sheep No clear pattern could be established relative to isolation of *S.* Typhimurium from different specific and type of examined RBC of different origin. Eight isolates of *S.* Typhimurium had ability to survive in serum after 1,3and 6 hours and could grow in serum denoting that they had a good ability to overcome body defense mechanism. Our data agree to great extant with **Atfeehy (2007)** who stated that *S.* Typhimurium isolated from ducks were positive to Congo red, survival& growth in serum and haemagglutination 100% but showing 100% & 66.66% MRHA to chicken and G.pig erythrocytes respectively.

All isolates of *S.* Typhimurium had ability to adhere and invade HEP-2 cell (causing death in cells with different degree) except one *S.* Typhimurium isolated from adult sheep could not invade HEP-2 cell. **Mohamed *et al*., (2012)** found that all *S.* Typhimurium isolated from buffalo calves were invasive to HEP-2 cell. The invasion of HEP-2 cells by salmonella serovars is a useful model for study of adhesive and invasive properties of this pathogen. The penetration of HEp-2 cells appears to results from endocytosis of the bacteria by the animal cells **(Jones *et al.,* 1982).**

Concerning mortality rate in mice of isolated *S.* Typhimurium from calves, adult cows, lambs and adult sheep were 53.3%, 40 %, 66.7 % and 60 % respectively. On the other hands the entrotoxigenicity were 66.7% and 0 % in *S*. Typhimurium isolated from calves and adult cows while were 66.7and 0% in *Salmonella* isolated

from lambs and adult sheep respectively. However, **Salman *et al*., (2007)** recorded the most of *Salmonella* isolated in diarrheic calves gave high degree of mortalities to mice and were enterotoxigenic by using infant mouse essay. **Also Ibrahim and El Gohary (2007)** found mortality rate in mice was (60%) in *S.* Typhimurium.

The results presented in Table (4) showed antimicrobial susceptibility patterns of 10 antimicrobial agents among salmonellae which showed variable results. It was found that all tested isolates were completely resistant to colistin sulphate and lincomycin.

Table 3. Virulence of *S.* Typhimurium isolated from diarrhoeic cattle and sheep.

Virulence tests	Calves 3**		Adult cow 1**		Lambs 3**		Adult sheep 1**		Total 8	
	No.	%*	No.	%*	No.	%*	No.	%*	No.	%**
Congred.	3	100	1	100	3	100	1	100	8	100
Haemagglutination:										
Bovine										
-MSHA	3	100	1	100	2	66.7	1	100	7	87.5
-MRHA	0	0	0	0	0	0	0	0	0	0
-HA	3	100	1	100	2	66.7	1	100	7	87.5
Sheep										
-MSHA	2	66.7	1	100	3	100	1	100	7	87.5
-MRHA	1	33.3	0	0	0	0	0	0	1	12.5
-HA	3	100	1	100	3	100	1	100	7	87.5
G.pig										
-MSHA	0	0	0	0	1	33.3	0	0	1	12.5
-MRHA	2	66.7	0	0	2	66.7	1	100	5	62.5
-HA	2	66.7	0	0	3	100	1	100	6	75.0
Chicken										
-MSHA	1	33.3	0	0	0	0	0	0	1	12.5
-MRHA	1	33.3	0	0	2	66.7	0	0	3	37.5
-HA	2	66.7	0	0	2	66.7	0	0	4	50.0
Serum resistance: A*Survival										
Serum.										
1hr.										
3hr.	3	100	1	100	3	100	1	100	8	100
6hr.	3	100	1	100	3	100	1	100	8	100
B*Growth Serum.	3	100	1	100	3	100	1	100	8	100
	3	100	1	100	3	100	1	100	8	100
Pathogencity test:										
HEP. 2 cell										
Adherence assay	3	100	1	100	3	100	1	100	8	100
Invasion assay	3	100	1	100	3	100	0	0	7	87.5
Mouse lethality test.	8/15	53.3	2/5	40	10/15	66.7	3/5	60	23/40	57.5
Entrotoxine production.	2	66.7	0	0	2	66.7	0	0	4	50.0

*The percentage was calculated according to the number of strains of each age except in pathogenicity in mice the percentage was calculated according to the number of each mouse used in each age group.
**Number of examined serovars.

On the other hand, gentamicin, flumequine and norfloxacin gave good susceptibility followed by ofloxacin. **Atfeehy (2007)** stated that *S.* Typhimurium were resistant to amoxicillin &flumequin, intermediate to ciprofloxacin, and sensitive to colistin sulphate, ofloxacin 100% and 66.66% to doxycyclin. Meanwhile, **Ibrahim and El Gohary (2007)** recorded that *S.* Typhimurium isolated from lambs and sheep were highly susceptible to ciprofloxacin and enrofloxacin then to flumequine then amikacin. On the other hand the lower susceptibility incidences were observed in streptomycin and cefadroxil. **Ahmed *et al.*, (2009)** isolated nine salmonellae from 220 fecal samples from neonatal calf diarrhea in Egypt, six of these showed multidrug resistance and the isolated serovar were *S.* Typhimurium and *S.* Enteritidis.

From the above mentioned results, it is concluded that the most prevalent serotyps were *S.* Typhimurium which have a different virulence markers enabled it to play a significant role in inducing diarrhea in calves, adult cows, lambs, and adult sheep and focus should be thrown to the use of antimicrobial agents in the field to prevent spread of salmonellae among animals and to human

Table 4. Drug susceptibility tests of *S.* Typhimurium isolated from diarrhoeic cattle and sheep.

Drug	Calves 3**						Adult cow 1**						Lambs 3**						Adult sheep 1**					
	S		I		R		S		I		R		S		I		R		S		I		R	
discs	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Amoxicillin (25µg)*	1	33.3	1	33.3	1	33.3	0	0	1	100	0	0	0	0	1	33.3	2	66.7	0	0	0	0	1	100
Ciprofloxacin (5µg)	2	66.7	1	33.3	0	0	0	0	1	100	0	0	2	66.7	1	33.3	0	0	1	100	0	0	0	0
Colistin sulphate (50µg)	0	0	0	0	3	100	0	0	0	0	1	100	0	0	0	0	3	100	0	0	0	0	1	100
Enrofloxacin (5µg)	2	66.7	1	33.3	0	0	0	0	0	0	0	0	2	66.7	1	33.3	0	0	1	100	0	0	0	0
Flumequine (30µg)	2	66.7	1	33.3	0	0	1	100	0	0	0	0	2	66.7	1	33.3	0	0	1	100	0	0	0	0
Gentamicin (10µg)	3	100	0	0	0	0	1	100	0	0	0	0	2	66.7	1	33.3	0	0	1	100	0	0	0	0
Lincomycin (10µg)	0	0	0	0	3	100	0	0	1	100	0	0	0	0	0	0	3	100	0	0	0	0	1	100
Norfloxacin (10µg)	2	66.7	1	33.3	0	0	1	100	0	0	0	0	1	33.3	2	66.7	0	0	0	0	1	100	0	0
Ofloxacin (5µg)	2	66.7	1	33.3	0	0	1	100	0	0	0	0	2	66.7	1	33.3	0	0	1	100	0	0	0	0
Tetracycline (30µg).	0	0	2	66.7	1	33.3	0	0	0	0	1	0	0	0	1	33.3	2	66.7	0	0	1	100	0	0

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