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Major Gram-negative bacterial causes isolated from apparent Healthy and diarrheic foals in Egypt, prevalence, identification and antibiotic susceptibility profiles

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ABSTRACT

Diarrhea is one of the most significant illnesses affecting young foals and may be manifested in more than half of foals up to 3 months of age. Numerous infectious causes have been involved but bacterial pathogens are concerned. This study aimed to assess and identify the prime Gram-negative bacteriological etiology of Arabian horse foals' diarrhea in Egypt and to designate the antibiotic susceptibility profiles of the isolated microorganisms. Rectal swabs and internal organs were obtained from 216 foals (89 diarrheic and 127 apparently healthy), aged 1 week to 1 year old, reared in Arabian horse farms in Great Cairo, Egypt during a period extended from March 2022 to December 2022. Conventional bacteriological examination was performed using selective media persuaded by routine and advanced biochemical tests. The isolation displayed 648 bacterial isolates; 452 (68.6%) were Gram-negative with the mixed isolation representing about 28%. *Escherichia coli* constituted the most prevalent; 110 / 452 (24.34%); 65 in apparently healthy foals and 45 among diarrheic ones. The second was *Klebsiella pneumoniae* (72, 15.92%) at which 50, (15.67%) were found to expose the highest causative agent recovered from diarrheic foals. Imipenem, quinolones, and trimethoprim/sulfamethoxazole were the most effective versus Gram-negative species isolated from diarrheic foals while ampicillin and tetracycline were of no effect. The phenotypic antibiotic susceptibility test revealed a picture of multidrug resistance (MDR) as 111 isolates (28.71%) showed resistance to three or more antibiotics belonging to different groups. The results demonstrated that *Klebsiella pneumonia* was the highest MDR species (54.16%) followed by *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli* isolates by 43.9, 32.1 and 30.9 % respectively. In conclusion, Gram-negative bacteria constituted the major causative agents of diarrhea in the Arabian foals. The most effective antibacterial drugs were imipenem, quinolones, and trimethoprim/ sulfamethoxazole. The issue of the existence of multidrug resistance isolates should be considered for proper therapy of foal diarrhea.

Introduction

Foal diarrhea is a global significant concern in equines and is reported to be the major common reason of death at a young age, up to 6 months of life, 20% of foals have been accounted to suffer from infectious diarrhea resulting in economic losses worldwide (Haq *et al*., 2018; Oliver-Espinosa, 2018).

The influenced foals display varied symptoms; colic, hypermotility, abdominal distension, watery to pasty, or even bloody diarrhea, frequent anorexia, and poor body condition (Mallicote *et al*., 2012). The cases usually proceeded to dehydrate rapidly and may reach severe dehydration manifested by sunken eyeballs and a prolonged skin tent. The bad consequences may occur; recumbence, coma, septic shock, and even death (Haq *et al*., 2017).

It is substantial to explore potential reasons and identify infectious agents to bypass losses. Commonly, a single causative agent is unusual; co-infection between infectious agents was more prevalent and may lead to more cruel gastrointestinal illnesses (Olivo *et al*., 2016; Uzal *et al*., 2022). It is found that about 50-60% of diarrheic newly born foals' cases are of bacterial origin; the most common infectious agents include Gram-negative bacilli; *Escherichia coli*, *Salmonella*, *Klebsiella*, and *Enterobacter* spp. (Ata *et al*., 2020; Hain-Saunders *et al*., 2022).

The use of broad-spectrum antibiotics in foal's diarrhea is common, but there is broad evidence that their random uncontrolled uses are associated with the emergence of multi, extensive, or even pan-drug resistance which threats public health. Consequently, the determination of the proper efficient antimicrobial agents versus trans- species-specific pathogens is substantial for valid therapy and maintains the one health approach (Das *et al*., 2020).

Therefore, the current study was conducted to identify the prime Gram-negative bacteria implemented in diarrhea among affected Arabian horse foals in Egyptian farms parallel to the apparently healthy ones. Additionally, the susceptibility of obtained bacterial isolates against antimicrobial agents was also determined.

Materials and methods

Ethical approval

The study followed the guidelines of the National Committee of National Research Centre, Giza, Egypt under the code Vet CU 03162023720.

Animal

A total number of 216 foals (89 diarrheic and 127 apparently healthy), aged 1 week to 1 year old, reared in an Arabian horse farm in Great Cairo, Egypt during the period extended from March to December 2022 were sampled in this study (Table 1). The diarrheic foals exhibited profuse diarrhea accompanied by an increase in body temperature, inappetence, depletion, depression, and colic, while some cases died suddenly. Multiple data about age, severity of the case, nature of the diarrhea, body temperature, type, and response to the used antibiotics were collected using a questionnaire.

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Isolation

Sampling

Rectal swabs were gathered from affected foals in Brain Heart Infusion (BHI) broth as a transport medium, stored in icebox and transported as quickly as possible to the laboratory for bacteriological examination. Internal organs including (the liver, spleen, kidneys, intestine, and mesenteric lymph nodes) were collected from 9 dead foals. The samples were gathered under aseptic conditions in separate polyethylene bags, labeled, and sent in an ice box to the laboratory.

Bacteriological cultures

The collected rectal swab samples were firstly inoculated into trypticase soya broth overnight at 37°C. The swabs were streaked onto the following cultural selective and specific media; MacConkey agar, sorbitol MacConkey (SMAC), eosin methylene blue agar (EMB), and *Pseudomonas* agar. For *Salmonella* isolation, fecal samples were inoculated into selective tubes containing tetrathionate broth and then incubated overnight in aerobic conditions at 37°C. After incubation, samples were cultured on Xylose Lysine Deoxycholate agar plates, Brilliant Green agar plates and incubated aerobically at 37°C for 24-48 hours.

Morphological characteristics

After incubation, the plates were noticed for the growth of bacterial colonies. The morphological appearance of colonies grown on MacConkey, EMB, and XLD indicated sugar fermentation. Colony morphology, color, size, elevation, and the status of hemolysis were recorded (Quinn *et al*., 2013). Representative colonies from culture-positive plates were subcultured for isolation of pure colonies and subjected to Gram's staining procedure and examined microscopically under oil emersion lens.

Identification

Biochemical identification

Pure colonies were subjected to further biochemical tests as oxidase, nitrate, urease, catalase, TSI (triple sugar iron agar), coagulase, indol, methyl red, Voges-Proskauer, citrate (IMVC), and sugar fermentation. These culture isolates were tested for determination of the presence of oxidase enzyme to differentiate between the oxidase-negative *Enterobacter*iaceae and other Gram-negative oxidase-positive bacteria (Quinn *et al*., 2013). Moreover, pure colonies were biochemically analyzed by API (Analytical Profile Index) systems such as API 20 E and API 20 NE Systems for members of *Enterobacter*iaceae and non-*Enterobacter*iaceae (BioMerieux, Marcy l'Etoile, France), according to manufacturer guidelines.

Species-level identification

The species level was further identified via an automated identification system; Vitek2 compact system Version 9.02 MIC Interpretation Guidelines 'according to the manufacturer instructions'(Vitek 2 product information, document 510769-4EN1.bioMe´rieux, Inc., Durham, NC.

In detail, sufficient numbers of morphologically similar colonies were transmitted to a saline tube; homogenous suspension was prepared against a density equivalent to a McFarland 0.50 -0.63 using DensiChek. Each isolate's suspension tube as well as a specific identification card was placed in the cassette, incubated, and read every 15 minutes via the instrument's software (Ling *et al*., 2001).

Antimicrobial susceptibility assay

The susceptibility of the isolates to various twenty antibiotic discs (Oxoid, UK) mentioned in Table 2 was achieved. The disc diffusion method using Muller Hinton agar was performed according to the Clinical and Laboratory Standard Institute (CLSI) recommendations (CLSI, 2020). The degree of susceptibility was determined by measuring the zone of growth inhibition produced by the diffusion of the antibiotic into the surrounding medium after the incubation at 37°C for 24 h under aerobic conditions. The results were interpreted according to the methods of Kassim *et al*. (2016).

Results

Bacteriological findings

A bacteriological examination of 216 samples obtained from 89 diarrheic foals and 127 apparently healthy ones revealed the ion of 452 isolates that showed Gram-negative reaction which exhibited either negative or positive oxidase test.

The negative oxidase test Gram-negative isolates cultivated on MacConkey agar were generally exposed smooth, flat, circular, and medium in size. The colonies were either lactose fermenter (appeared pink) or non-lactose fermenter (colorless). Suspected Gram-negative *E. coli* isolates displayed flat lactose fermenter pink circular colonies on MacConkey agar, and black with metallic green sheen colonies on EMB agar.

Klebsiella spp. isolates showed circular, mucoid pink or purple dark-centered lactose fermenter colonies on MacConkey agar, also the same colonial appearance was presented on EMB agar. Suspected *Enterobacter* spp. were late lactose fermenter after 48 hours and the colonies were light pink, mucoid, and smaller than *Klebsiella* spp.

Furthermore the non-lactose fermenter, Gram-negative isolates which represented presumptive *Proteus* spp. (characteristic swarming phenomena) and *Salmonella* spp. displayed black colonies on XLD agar plates.

The other non-lactose fermenter positive oxidase tested isolates that produced pigmented colonies; suspected *Pseudomonas* spp. were transferred to nutrient agar and subcultured more than one time to obtain pure cultures which appear with characteristic phenomena that production of greenish pigmentation.

Regarding to the colonial differentiation and conventional biochemical reactions; the Gram-negative isolates (452 isolates) in both diarrhe-

Table 1. The number of examined apparently healthy and diarrheic foals.

	Age						
	1 week – 3 months			3-6 months		6-12 months	
Foals	Apparent Healthy	Diarrheic		Apparent Healthy	Diarrheic	Apparent Healthy	Diarrheic
		Life	died				
No.	25	45	9	62	25	40	10
Sum	25	54		87		50	
	79						
Total	216						

ic and apparently healthy foals were categorized into seven genera in addition to unidentified 29 isolates. These genera were Escherichia spp. (114 isolates), *Klebsiella* spp. (91 isolates), *Proteus* spp. (63 isolates), *Enterobacter* spp. (56 isolates), *Pseudomonas* spp. (47 isolates), *Salmonella* spp. (37 isolates), and *Citrobacter* spp. (15 isolates) as shown in Table 3.

Species-level identification

The species-level identification via API and Vitek2 compact system was obtained as demonstrated in Table 4.

Antibacterial sensitivity phenotyping test for the identified isolates

The phenotypic susceptibility profiles of obtained isolates versus the 20 tested antibiotics were shown in Figs. 1-6.

The results extracted from the figures revealed the profiles of different isolates to the tested twenty antibiotics; *Escherichia coli* isolates were completely resistant to ampicillin, neomycin, novobiocin, and tetracycline (100%) while most susceptible to imipenem (52.7%), trimethoprim/sulphamethoxazole (34.5%) and quinolones (30%). *Klebsiella pneumonia* as well as *Klebsiella oxytoca* isolates were strictly resistant to the same previous four antibiotics besides both ceftazidime and erythromycin while were very susceptible to both imipenem and trimethoprim/sulphamethoxazole (66.7%) and amikacin (54.2%). *Salmonella enterica* isolates were completely resistant to many antibiotics including amoxicillin/ clavulanic acid, ampicillin/sulbactam, cephalosporins, and aminoglycosides, but susceptible to trimethoprim/sulfamethoxazole (78.6%) then amikacin and cefoxitin (67.9%) for each. Isolates belonging to *Enterobacter* spp. were generally resistant to aminoglycosides and sensitive to imipenem (73.7%) and quinolones (55%). *Citrobacter freundii* isolates were completely sen-

Table 2. The used antibiotic discs.

Antibacterial disc	Abbreviations	Concentration	Antibacterial disc	Abbreviations	Concentration
Amikacin	AK	30 µg	Erythromycin	E	15 µg
Ampicillin	AM	10 µg	Imipenem	IPM	10 µg
Amoxicillin/Clavulanic acid	AMC	30 µg	Levofloxacin	LEV	5 µg
Ampicillin/Sulbactam	SAM	10/10µg	Nalidixic acid	NA	30 µg
Cefoxitin	FOX	10 µg	Neomycin	N	30 µg
Ceftazidime	CAZ	30 µg	Penicillin G	P	10 IU
Ceftriaxone	CRO	30 µg	Rifampicin	RD	5 µg
Cefotaxime	CTX	30 µg	Streptomycin	S	10 µg
Ciprofloxacin	CIP	5 µg	Trimethoprim/sulfamethoxazole	SXT	25 µg
Gentamicin	CN	10 µg	Tetracycline	TE	30 µg

Table 3. type and number of suspected Gram negative isolates.

Identified bacteria	No. of suspected isolates	Percentage	Total no. of isolates
Escherichia spp.	114	25.22%	452
Klebsiella spp.	91	20.14%	
Proteus spp.	63	13.94%	
Enterobacter spp.	56	12.38%	
Pseudomonas spp.	47	10.40%	
Salmonella spp	37	8.18%	
Citrobacter spp.	15	3.33%	
Unidentified spp.	29	6.41%	

Table 4. species typing of the isolated Gram negative bacteria.

Animal	Apparently healthy foals (n.= 127)		Diarrheic foals (n.= 89)		Total Sum (n.= 216)	
Bacterial isolates	No	%	No	%	No	%
Gram negative bacteria						
Escherichia coli	65	48.87	45	14.1	110	24.34
Klebsiella pneumoniae	22	16.54	50	15.67	72	15.92
Klebsiella oxytoca	5	3.76	12	3.76	17	3.76
Salmonella enterica	-	-	28	8.77	28	6.19
Enterobacter aerogenes	29	21.8	9	2.82	38	8.4
Enterobacter cloacae	12	9.02	2	0.62	14	3.09
Citrobacter freundii	-	-	11	3.44	11	2.43
Proteus mirabilis	29	21.8	6	1.88	35	7.74
Proteus vulgaris	16	12.3	3	0.94	19	4. 20
Proteus penneri	7	5.26	2	0.62	9	1.9
Pseudomonas aeruginosa	-	-	41	12.85	41	9.07
Unidentified	23	17.29	35	10.97	58	12.83
Sum of Gram –ve isolates	133		319		452	

sitive to most tested antibiotics except tetracyclines and erythromycin contrarily, *Pseudomonas aeruginosa* isolates were strictly resistant to most antibiotics except imipenem.

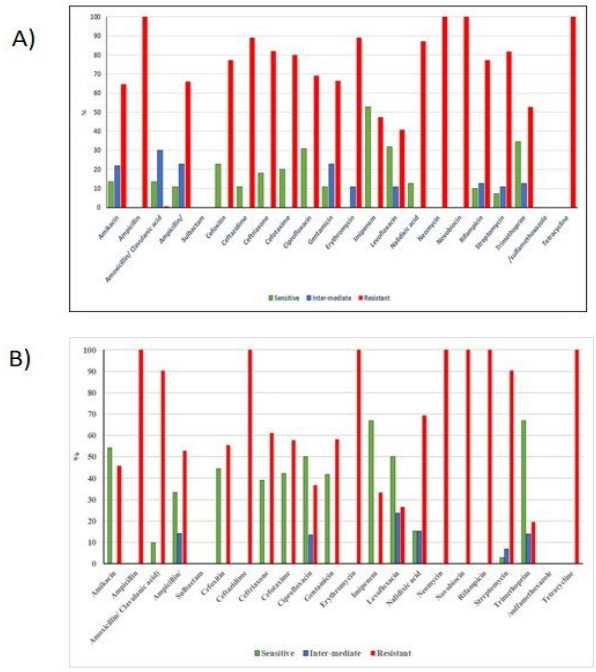

Fig. 1. A) Susceptibility of *Escherichia coli* isolates to the antibacterial agents. B) Susceptibility of *Klebsiella pneumonia* isolates to the antibacterial agents.

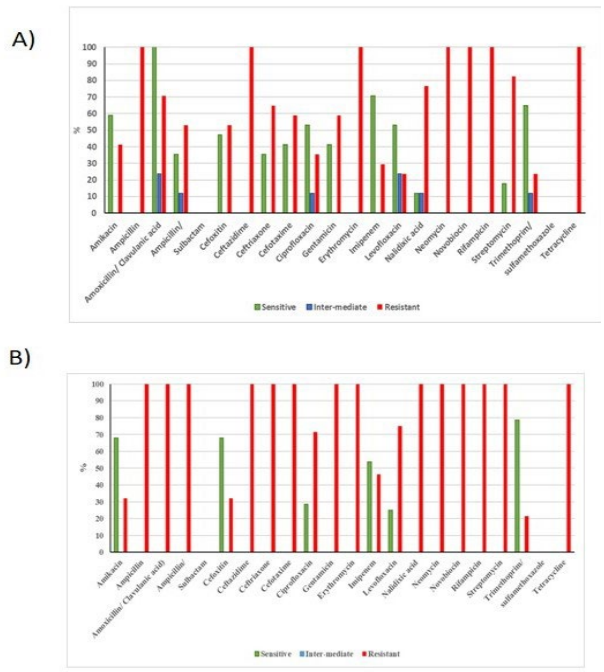

Fig. 2. A) Susceptibility of *Klebsiella oxytoca* isolates to the antibacterial agents. B) Susceptibility of *Salmonella enterica* isolates to the antibacterial agents.

Finally, isolates belonging to *Proteus* spp. were generally susceptible to large-scale tested antibiotics.

The results obtained from the phenotypic antibiotic sensitivity test exposed 111 isolates (28.71%) were resistant to three or more different antibiotic groups giving the picture of multidrug resistance (MDR). The results shown in Figure 7A, demonstrated that *Klebsiella pneumonia* was the highest MDR species (54.16%) followed by *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli* isolates by 43.9, 32.1, and 30.9 % respectively.

Results obtained from Figure 7B, indicated that the most effective antibacterial drug against the isolated Gram-negative bacteria was imipenem (67.4%) followed by trimethoprim/sulphamethoxazole, cefoxitin, ciprofloxacin then amikacin as 43.5, 43.2, 42.6 and 40.4% respectively. On the other side tetracycline (0.5%), ampicillin (1.3%), and novobiocin (4.3%) possessed the least efficiency (Fig. 8).

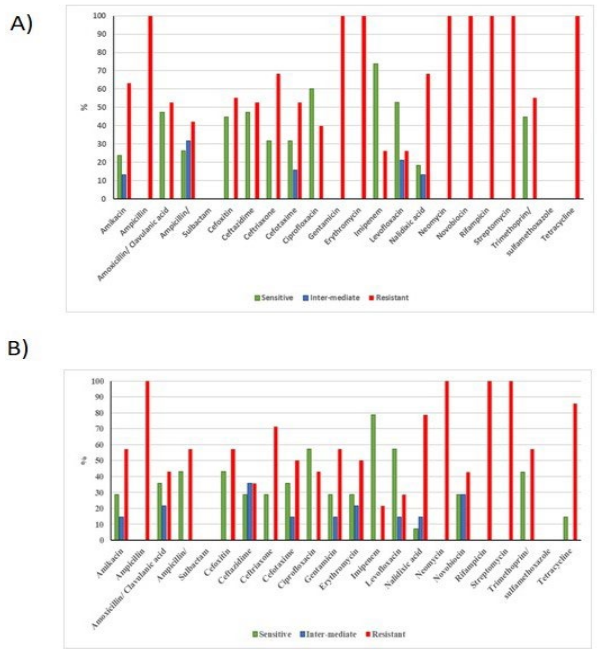

Fig. 3. A) Susceptibility of *Enterobacter* aerogens isolates to the antibacterial agents. B) Susceptibility of *Enterobacter cloacae* isolates to the antibacterial agents.

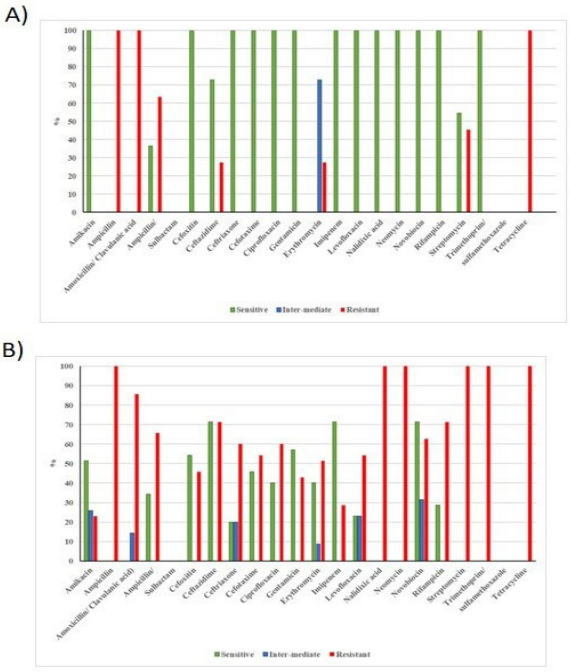

Fig. 4. A) Susceptibility *Citrobacter freundii* isolates to the antibacterial agents. B) Susceptibility of *Proteus mirabilis* isolates to the antibacterial agents.

Discussion

Diarrhea is a prime reason for morbidity and mortality in the young foal, which is accompanied by a systemic inflammatory response syndrome. Foals with enteritis develop different degrees of endotoxemia and complain of a number of metabolic complexities (Magdesian, 2005).

Our results agreed with other surveys that reported Gram-negative bacteria as common causes of diarrhea in foals (Hollis *et al*., 2008), Enterotoxigenic *E. coli* ((Holland *et al*., 1989), *Salmonella* spp., and Enterococcus spp. (Traub-Dargatz and Besser, 2007).

The current study elucidated the isolation of *Escherichia coli* by 25.22% which coincided with that obtained by Olivo *et al.* (2016); 30% in diarrheic foals and 25% in healthy ones. Our study determined the isolation of *Klebsiella* spp. by 20.14% and this result was identical to that

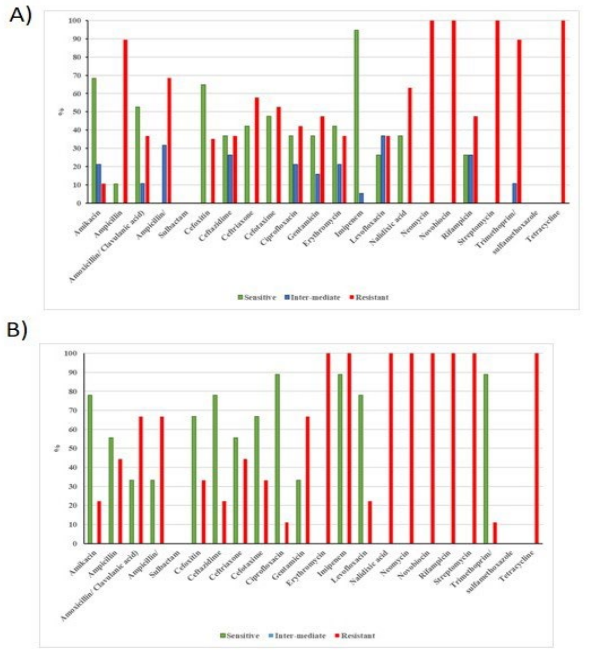

Fig. 5. A) Susceptibility of *Proteus vulgaris* isolates to the antibacterial agents. B) Susceptibility of *Proteus penneri* isolates to the antibacterial agents.

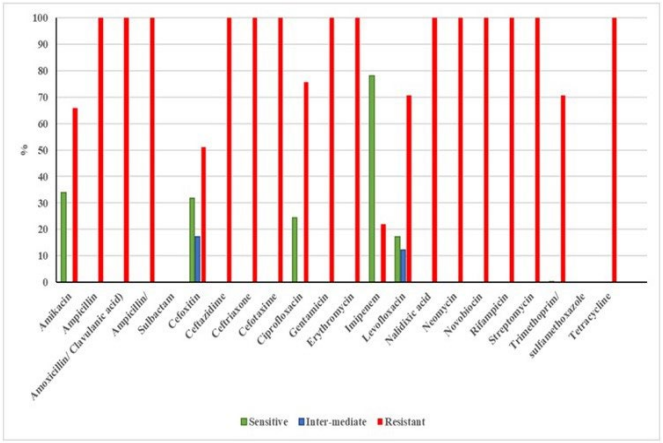

Fig. 6. Susceptibility of *Pseudomonas aeruginosa* isolates to the antibacterial agents.

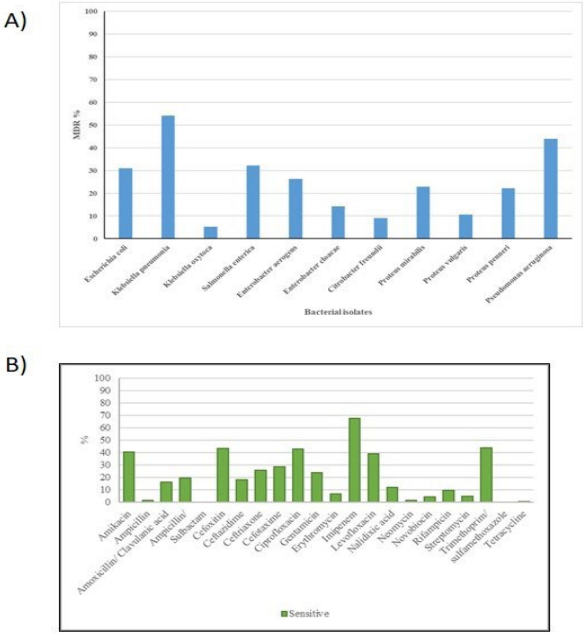

Fig. 7. A) Multidrug resistance % of different Gram-negative bacteria isolated from both diarrheic and apparently healthy foals. B) The susceptibility % to the twenty tested antibacterial drugs among different Gram-negative bacteria isolated from both diarrheic and apparently healthy foals.

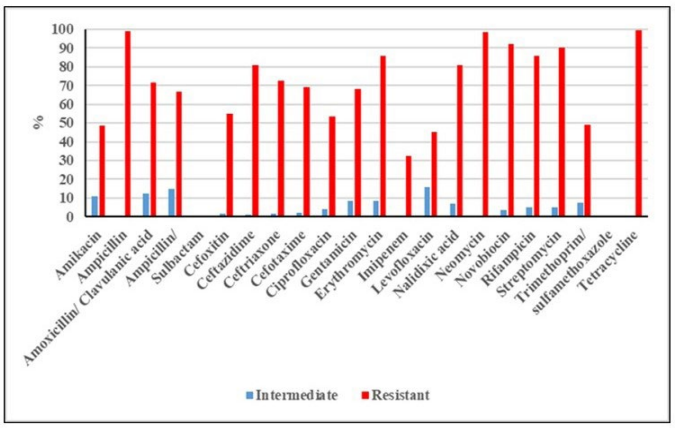

Fig. 8. Resistance and intermediate resistance % of different Gram negative bacteria isolated from both diarrheic and apparently healthy foals to the twenty tested antibacterial drugs

reported by Samir *et al.* (2022); 20% was obtained from diarrheic horses. On other hand our *Klebsiella* spp. isolation percentage was higher than that mentioned by Ribeiro *et al.* (2022); 14.1%.

Hollis *et al.* (2008) reported the isolation of Gram-negative organisms (57%) in diarrheic foals; *Escherichia coli* (10.7%), *Salmonella* spp. (10.7%), *Klebsiella* spp. (5.3%), in addition to *Citrobacter freundii*, and *Enterobacter* spp. each has single isolates.

Our results agreed with several studies; Hollis *et al.* (2008) tested *E. coli* isolates that recovered from diarrheic foals less than one month of age and mentioned their sensitivity to amikacin, gentamicin, and imipenem. Reshadi *et al.* (2021) mentioned that diarrheagenic *E. coli* isolated from riding horses in Germany showed resistance to β-lactams (ampicillin and cephalosporins) then aminoglycoside and trimethoprim/sulphamethoxazole. *E. coli* isolates obtained from sports horses revealed resistance to ampicillin and tetracycline and were highly susceptible to gentamycin and quinolones (Wongtawan *et al.*, 2022).

de Lagarde *et al.* (2019) mentioned that approximately forty-four percent of horses in France shed MDR *E. coli*. Their prevalent resistance was against ampicillin, amoxicillin/clavulanic acid streptomycin. Parallel to this study in Canada, de Lagarde *et al.* (2020) demonstrated a little higher ratio; 46.3%. The shed MDR *E. coli* were non-susceptible to the same three antibiotics.

Regarding *K. pneumonia* isolates were mostly resistant to ampicillin, ceftazidime, erythromycin, neomycin, novobiocin, and tetracycline (100%), this result was slightly agreed with that determined by Ribeiro *et al.* (2022) which reported high resistance to ampicillin (91.8%), but susceptible to trimethoprim/sulphamethoxazole. In contrast to our results, most isolates of *Salmonella* spp. were sensitive to the great extent of tested antimicrobials (Awosile *et al.*, 2018).

Not surprising that *Pseudomonas aeruginosa* isolates were totally resistant to 14 antibiotic kinds, the isolates were sensitive only to five antibiotics: imipenem then cefoxitin, amikacin, ciprofloxacin, and levofloxacin. Previous studies mentioned that P. aeruginosa has a unique complicated antibiotic resistance mode and is usually considered a multidrug-resistant organism (Gajdács *et al.*, 2020).

Conclusion

The prime Gram-negative species that implemented in foal diarrhea were *E. coli*, *Klebsiella* spp., *Salmonella enterica*, *Proteus* species, *Enterobacter* species, *Pseudomonas* spp. The most efficient antibacterial drugs are imipenem trimethoprim/sulphamethoxazole, amikacin, and quinolones, while cephalosporins and aminoglycosides were variable in their action. On the other hand, great extents of the isolated species are strictly highly resistant to ampicillin, neomycin, novobiocin, and tetracyclines. The antibiotic susceptibility profile of different Gram-negative species isolates is greatly useful in foal diarrhea therapy protocol.

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Conflict of interest

The authors declare that they have no conflict of interest.

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