Multiple antibiotic resistance of biotypes *Helicobacter pylori* isolates recovered from sero-examined sheep and felines in Upper Egypt

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Abstract:

That study showed the multiple antibiotic resistance of variant biotypes of Helicobacter pylori isolates from felines and sheep in Upper Egypt. Total one hundred and thirty- five isolates were recovered from (6 stomach and 46 stool) of 52 felines and (66 gastric and 17 milk) of 83 sheep samples in Hurghada, Luxor, and Sohag provinces. Stool samples of felines and serum samples of sheep were sero-examined using a stool antigen test and latex agglutination test of *H. pylori*, respectively in total percent 67.3% for enriching felines and sheep specimens, respectively in sterile tubes of supplemented or un-supplemented 5ml thioglycolate broth with 10 µl hemin within 36 hours under microaerophilic condition at 37^{c} then will be cultivated onto filled plates from 500 ml blood brain heart agar supplemented with 135 µl urea 40% solution, novobiocin 500 µl, vancomycin 25 µl, and amphotericin B 350 µl for 5 days and differentiated microscopically upon gram negative stain, and biochemically upon positive oxidase, and variable enzymatic activity of urease and nitrate reduction that be confirmed by *16srRNA H. pylori* polymerase chain reaction in total percent 8.8%. Four *H. pylori* isolates in each province were detected by antimicrobial susceptibility test to report the highest multiple antibiotic resistance MAR of *H. pylori* isolates (1.0) plus (0.643) & (0.857) among wide ratio of urease & nitrate reduction

isolates U: N (1:1.8) were recovered from Hurghada. But equal ratio of U: N isolates included the lowest MAR of *H. pylori* isolates (0.07) plus (0.5), (0.286) and (0.714) were recovered from Sohag.

Keywords: Antimicrobial susceptibility test (AST), multiple antibiotic resistance, *H. pylori*, nitrate, Upper Egypt, sheep and felines.

Introduction

Helicobacter pylori is the primary microorganism species verified to cause cancer and is assessed as a group I carcinogen by the International Agency for Research on Cancer **(*Testerman & Morris*, 2014).** Role of *H. pylori* in idiopathic thrombocytopenic purpura and iron deficiency anemia is well documented, in addition to the principal cause of gastric adenocarcinoma (***Tsay & Hsu*, 2018**). As a history of *Ascaris* infection and *Mycobacterium bovis* BCG immunization ***Fernando et al.* (2001**) that increasing trans-exudation of serum components as weak urease activity (HP-W) and strong urease activity (HP-S) as *H. pylori* HP0013 ***Sarraseca et al*. (1998)** sensitivity against antibiotics (***Leitsch*, 2019**). Antibiotic resistance of *H. pylori* hampers the success of eradication and in recent years shows an increase multidrug resistance (MDR) worldwide (***Rokkas & Ekmektzoglou*, 2023**) was associated with bacterial factors such as outer membrane protein (***Zhu et al.*, 2021).** *H. pylori* infection by cats, dogs, and sheep to humans given an unproved zoonotic pathway to a human and non-human primate through bacteria-bacteria reaction ***Mladenova-Hristova et al.* (2017)** by outer membrane of *H. pylori* which is difficulty isolated free of inner membrane ***8. Doig et al.* (1997).** *H. pylori* genome and its virulence gene association to their geographic origin and pathogenicity as urease gene was postulated (***Rodriguez et al*. 2021**). Active site of ureases of *H. pylori* is conserved among living organisms upon optimizing urease activity by acting of vacuolating cytotoxin associated gene *vac*A as passive urea transporters to favor *H. pylori* infectivity ***Tombola et al.* (2001)** upon nickel repellant urea ***Sanders et al.* (2013)** or ability *H. pylori* to attract and respond to environmental iron concentrations as critical to survive ***Haley & Gaddy*** (**2015)** through natural urease inhibitors as soil microbiota enriched with NO3, present in extracts of clinical as pure compounds may go to eutrophication reduction of animal populations and threat to human health **Modolo *et al*. (2015**). Survival and transmissible *H. pylori* acquiring urease activity from neighboring bacteria in generation of a pH neutral micro-environment when suitable amount urease hydrolyses into ammonia and C02 ***Dunn & Phadnis* (1998)** by endocytosis of outer membrane

proteins which were investigated by urease and nitrate reduction test under optimal conditions (atmosphere 5% o_2, 80-90% and 5-10% co_2), the humidity (96-100%) and temperature 37^{o}c for evaluation of the nitrate-nitrite-NO pathway (***Graham & Miftahussurur*, 2018).** *H. pylori* survives in acidic environment where ammonium is toxic by producing abundant quantities of urease from subpopulation of *H. pylori* that contains cytoplasmic urease only in vivo, unable to bind free urease with specific antibodies secretory *Ig*A (***Krishnamurthy et al.*, 1998*).*** Prevalence of *H. pylori* antibodies in farm animals and the chicken was 70.3%, 68%, 96.4% and, 88.9% in cattle, sheep, goats, and chickens, respectively in Sudan ***Mohammed et al*. (2014)** that may cause an oncogenic transformation in transgenic animals through severe malignant lesions ***Knorr et al.* (2019)** in the future in nearly closed regions to Sudan such as Sohag as study area. Slaughterhouse tools are sources for multidrug-resistant and virulent pathogenic microorganisms which are a serious health problem (***Al-Kadmy et al.*, 2023)**. Highly seroprevalence *H. pylori* from felines which was considered as anthropozoonosis ***Cittelly et al.* (2002),** especially in Hurghada where *H. pylori* survive better in deep ground and sea water ***Konishi et al.* (2007)** to represent different sources of transmission as hygienic and chemical sources of pipes water ***Haroun et al.* (2011).** Colonization of *H. pylori* occurs through long incubation for culture that be used to determine the rate of the severity of disease if be related with *H. pylori* or cocultured with its bacterial competitor from the stomach tissue such under 40c ***Sánchez-Alonzo et al.* (2020)**, as similar to dry climate in occupational cities have pet clinics such as Luxor**.** In such stage of infection to gain therapeutic and economic targets ***Stevenson et al.* (2000)** for such region, using the Epsilometer (E-test) as a quantitive disc diffusion antibiotic susceptibility testing method that was concluded by ***Thyagarajan et al.* (2003)** as pattern of single and multiple resistance at respective center. Misuse of antimicrobials in food animals by 67 % from 2010 to 2030 against such *H. pylori*, endanger the health of both humans and animals in middle-income countries (***Anderson et al*.,, 2020**). Besides, the routine application of the antimicrobial susceptibility testing to detect the antibiotic of choice as well as the screening of the emerging Multidrug-resistant MDR strains. Several recent epidemiological investigations revealed the occurrence of MDR (resistant to ≥ 1 agent in ≥ 3 antimicrobial classes) bacterial pathogens from different origins (***Mumbo et al.* 2023**). For follow up of eradication therapy in such region, partial sequence of *16srRNA* of conserved regions ***Patel et al.* (2013)** or whole genome sequence

(WGS) of antimicrobial susceptibility test (AST) of suspected bacteria ***Pelegrin et al.* (2021**) is the most progressively genotypic methods of slowly growing microaerophilic bacteria as *H. pylori***.**

Materials &Methods

The animal requirements were approved from the Research Ethics Committee, Faculty of veterinary medicine, Suez Canal University (**Registration number**: **2016100)**.

Sampling and study area: During summer in 2017 to spring of 2021, 52 felines (6 stomach and 46 stool) and 83 sheep (66 gastric and 17 milk) were collected from Hurghada, Luxor, and Sohag provinces. Gastric samples of felines were collected from Animal care hospital according to euthanasia cases, in addition to stool samples were collected under permission of owner felines and clinicians as well as happen in Blue Moon pet clinics in Hurghada. Gastric samples of sheep were collected from slaughter houses of Hurghada and house market in Sohag.

Serological examination: Stool samples of felines and serum samples of sheep were examined by a stool antigen test **(Abon Biopharm)** and latex agglutination test **(Abon Biopharma (Hangzhou)., China),** respectively ***(Sabbagh et al*., 2019)**.

Isolation: Under aseptic condition, enriching samples were inoculated for 36 – 48 hours onto thioglycolate broth **(Himedia) India)** supplemented with urea ***Snell et al.* (1999)** and haemin ***Al Sulami et al.* (2008) (chlorid) (Roth.co.) Australia)** or BHI broth **(Oxoid) UK,** with no haemin as supplement under microaerophilic conditions using CampyGen gas kit (10% co2, 85%N2) (**Oxoid) (CN 0035A)** inside Jar 3.5 liter or 2.5 liter then reincubated for 5 days at 37c on BHI agar supplemented with antibiotics (vancomycin **(EMC. UK.)** and amphotericin B **(Astellas Pharma. US) (Agharid- Manssour & Ahmed 2008)**.

Biochemical differentiation: One hundred and thirty- five isolates out of totally collected samples were differentiated biochemically ***Harper et al.* (2003)** by oxidase **(Oxoid)**, catalase reagents**,** urease media (**HKM) Guangdong Huankai.** and nitrate reduction media (Peptone) **(HiMedia). India *Watt et al.* (1996),** using KNo3 reagent composed of (Potassium Nitrate **(Nasco). US** in weight 0.2 g dissolved into 1 ml D.W**)** to be characterized by gram staining (**Himedia) (*Park et al.*, 1984).**

DNA extraction of *H. pylori*: After overnight culture on brain heart infusion agar plates of all positive oxidase, catalase, urease and negative nitrate reduction in addition to some gram-negative rods or coccus have negative urease or positive nitrate reduction activity were detected using materials of DNA extraction from isolates by (**QIa amp Kit) *Chattopadhyay et al*.**

(2004), one or two colonies were suspended in an Eppendorf tube with 20 ml of sterile phosphate buffered saline and vortexed vigorously for 2 minutes. The tubes were boiled in a water bath for 15 minutes, cooled in ice, and centrifuged a 13000 g for 1 minute. The supernatant was transferred to another tube from which 1uL was used as the template for DNA amplification.

PCR detection of *16srRNA H. pylori*: **Gene JeT Genomic** used for DNA purification ***Chattopadhyay et al.* (2004),** including 50 µL of PCR Master Mix (**EzWay, Koma Biotech, Seoul, Korea),** composed of (5uL 10x buffer + MgCL2, 2mMdNTP, 2 uni Taq DNA polymerase) contained 100 ng of the extracted DNA and 25 pm of primer as shown in **table (A)** for amplification in thermal cycler **(Eppendorf, Hamburg, Germany)** at PCR conditions ***Tiwari et al.* (2007)** consisted of an initial denaturation of target DNA at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 30 s, primer annealing at 53°C for 30 s, and extension at 72°C for 45 s. The final cycle included extension for 5 min at 72°C to ensure full extension of the *16srRNA* product using 2 % agarose gel electrophoresis in 1 TBE buffer stained with ethidium bromide to be evaluated on a UV transilluminator. The *16srRNA* gene PCR gene product was (522) bp and all data were examined using Ladder 100bp ***Sambrook et al.* (1989)** (**Fermentas).**

Antimicrobial susceptibility test (AST): According to the guidelines stipulated by ***"NCCLS" Standards Laboratory Clinical for Committee National susceptibility antimicrobial for standards performance* (2001)**, the fourteen discs antibiotics (**Basingstoke, limited, Oxoid Hampshire, UK)** were over placed the surface of inoculated plate by using Plate agar method of nutrient agar as a substrate for growth of the tested bacteria for its antibiotic sensitivity by the single diffusion method ***Chen et al.* (2017)**. Moreover, the plate was incubated at suitable temperature 25 ^{0}c for 2-7 days and checked for the growth of the bacterium around the antibiotic disc which was demonstrated as well as the diameter of the zones of inhibition for the tested strains, the antimicrobial discs and their concentrations in **table (B)** for determination of multiple antibiotic resistance (MAR) index by using the formula MAR = No. of resistance \Total No. of tested antibiotics ***Singh et al.* (2010)** where isolates classified as intermediate were considered sensitive for MAR index).

Table (A): *Primers were used for identification of antigenic virulence of H. pylori isolates by 16srRNA and multiplex PCR.*

Primer	Sequence	Size	References
16srRNA	F5 GCGCAATCAGCGTCAGGTAATG3	522bp	**Hoshina et al. (1990).**
	R5 GCGCAATCAGCGTCAGGTAATG3		

Table (B): Antimicrobial discs, concentration and interpretation of their action on *H. pylori* isolates

Antimicrobial agent	disc content (µg)	Resistant (mm)	Intermediate (mm)	Susceptible (mm)
Cefazoline	30	10 or less	11-14	15 or more
Gentamicin	10	12 or less	13-14	15 or more
Tetracycline	30	14 or less	15-18	19 or more
Clarithromycin	15	10 or less	11-12	13 or more
Metronidazole	50	16 or less	17-19	20 or more
Levofloxacillin	5	18 or less	19-21	22 or more
Imipenem	30	18 or less	19-21	18 or more
Cephalothin	30	14 or less	15-17	18 or more
Amoxicillin	5	14 or less	15-18	11 or more
Ciprofloxacin	30	14 or less	15-19	20 or more
Amikacin	30	12 or less	13-15	16 or more
Penicillin G	10IU	20 or less	21-28	29 or more
Nalidixic acid	30	13 or less	14-18	19
Rifampicin	5	12 or less	13-15	16 or more

Results

Table (1) shows one hundred and thirty-five sero-examined animal species recovered 106 seropositive samples including; 35 felines (32 stool and 3 gastric) plus 71 gastric sheep (58 gastric and 13 milk), and 29 seronegative animals including; 17 felines samples (13 stool and 4 gastric) plus 12 sheep samples (10 gastric and 2 milk), collected from total eighty -three sheep and fifty-two felines that cultivated traditionally to be differentiated biochemically by urease and nitrate reduction test, providing +ve results urease and reduction of nitrate U & N (36 & 44) and negative results (99 & 91), respectively.

From seropositive and seronegative animals, specimens belong positive U & N into (17 & 23) and (19 & 21) respectively, and negative U & N was isolated into (89 & 83) and (10 & 8) respectively, divided into

positive U: N (1.3: 1) and (1:1.1) and negative U: N (1:0.9) and (1:0.8).

In Hurghada, unbalanced U: N positive and negative ratio (1:8 or 1:1.2) and (1:0.7 or 1:0.4) was divided from (7:13 or 11:14) and (25:19 or 5:2) isolates, respectively from 20 seropositive, 4 seronegative sheep and 12 negative felines, which was detected by PCR from *16srRNA H. pylori* in percent 16.6% and 50% from each two seronegative felines and sheep, respectively.

In Luxor, among positive or negative biochemical reaction isolates that included 4 confirmed isolates by PCR in percent 17.3%, nearby equal U: N ratio ~:0 or 1:1.25 from U & N (1 & 0) and (4 & 5) isolates from five seronegative felines, and equal U: N 5:5 or 18:18 from 23 seropositive felines respectively, was isolated. In Sohag, equal U & N isolates either be positive or negative (5 & 5 or 7 & 7) or (46 & 46 or 1&1), respectively from 51 seropositive sheep or 8 seronegative sheep, which was confirmed by PCR from 3 seropositive and 1 seronegative sheep in percent 5.8% & 1.25%, respectively.

Twelve isolates in percent 8.8% were confirmed from felines in 11.5% and sheep in 7.2%, divided into six isolates from each six felines and sheep, grouped into 6.6% seropositive (4.2% sheep & 11.4% felines) and 17.2% seronegative (25% sheep & 11.7% felines), including 7 & 5 isolates, respectively from 3 seropositive or seronegative sheep, and (4 and 2) felines either be seropositive seronegative, respectively.

Table (2) shows three isolates of each 71seropositive or 12 seronegative from 83 sheep were confirmed by PCR in percent 3.6%, forming 6 isolates in percent 7.2%, grouped from 85.5% seropositive and 14.4% seronegative through urease and nitritic reduction isolates (15 & 18) or (68 & 60), respectively into positive U &N isolates ratio (6 :12) or (9:6) or negative U & N isolates ratio (65:59) or (3:1), respectively divided into +ve U:N (1:2) or (1: 0.6) or -ve U:N (1.1:1) or (1:0.3) forming totally U:N ratio (1:1.2) or (1:0.8), respectively.

Equal U: N isolates ratio in Sohag for sheep was detected from 86.4% (51) seropositive and 13.7% (8) seronegative sheep, respectively in total (59) 6.7%, forming 4 isolates from 3 seropositive sheep and one seronegative in percent 5.8% and 1.25% with MAR (0.714, 0.5 & 0.071) and MAR (0.286), respectively.

Un-balaanced U: N isolates ratio in Hurghada either be positive or negative (3:11) or (21:13) which was differentiated two isolates (50%) from (4) seronegative sheep 16.7% in percent 8.3% from 24 sheep, including MAR (1/0 & 0.643) among isolates +ve U: N ratio (2:4) or -ve U:N ratio (2:0) that divided into (1:2) or (1: ∞). Also 20 seropositive sheep in percent 83.3% have un equal balanced U: N isolates ratio +ve: (1:7) or -ve (19:13),

divided into (0.1:1) or (1:0.7) with 0% isolation.

Table (3):

Positive or negative U: N isolates (1:1) or (1:1.1) were recovered, respectively from seropositive felines where seroprevalence felines in Hurghada have 50% (12\24) with no confirmed *H. pylori* isolates, but +ve or -ve U: N (1:1) or (1:0.6), respectively from seronegative felines was detected one isolate with MAR 0.857 among total nearly equal U : N isolates ratio +ve (15:16) and -ve (9:8) that divided into (1:1.06) and (1: 0.8), respectively.

Seroprevalence in Luxor was 82.1% (23\28) belong equal U: N isolates ratio +ve (5:5) or -ve (18:18) for felines which was detected in percent 17.3% from seropositive felines in total 14.2% (4\28) with MAR (0.857, 0.428 & 0.214). Unbalanced U: N from 5 seronegative felines in percent 17.9% which recovered negative U: N isolates ratio (4 & 5) or positive U: N isolates (1:0) that divided into (1:1.25) or (1: ∞), respectively have no *H. pylori* isolates. Total U: N isolates ratio was almost equal in Luxor if positive or negative 6:5 or 22:23, respectively that divided into (1:0.8) or (1:1.04), or in sum of felines to isolate *H. pylori* in percent 11.5% (6\52) divided into 7.6% (4) and 3.8% (2) from 35 seropositive or 17 seronegative felines, in percent 67.3% and 32.7%, respectively.

Table (4) represent the highest MAR (0.857) of two *H. pylori* isolates from stool of seronegative felines which were investigated from 3 constipated and 2 pan-leukopenia felines in pet clinics to be cultivated biochemically producing positive urease and nitrate reduction reaction when while one negative nitrate reduction isolate was associated with seropositive felines that diagnosed as one diarrhea associated gastritis from 20 felines, with MAR 0.857 which decrease in descending follow as 0.428, 0.428 & 0.214 to recovery one highly urease *H. pylori* isolate from each 4 normal, one prolapsed uterus and one suddenly dead felines non clinical cases.

Table (5) compared the nitritic associated urease reaction between the lowest MAR 0.071 of one *H. pylori* isolate recovered from congested gastric seropositive sheep with negative nitrate reduction, and the highest MAR (1.0) of one *H. pylori* isolate recovered from normal gastric seronegative sheep with positive nitrate reaction but negative urease reaction, in dissimilarity to other isolate from milk of negative delivered sheep with moderate low MAR (0.286) with positive urease and nitritic reaction together. Low MAR increase in ascending following (0.5, 0.643 & 0.714) from 2 seropositive of congested and normal gastric sheep, respectively and congested gastric of one seronegative sheep, respectively that was identified with positive urease and negative nitritc reaction.

Table (6) shows highest sensitivity against amoxicillin and tetracycline in percent (100 & 83.3%) start with

highest antibiotic resistance 0.857 against *H. pylori* isolates from stool of seronegative constipated and pan-leukopenia felines, that decreased in percent 50 % with testing levofloxacin discs against diarrheal feline with the same MAR 0.857. Followed by sensitivity rifampicin, metronidazole clarithromycin, amikacin, and cephalothin in the following (33.3, 50, 33.3, 33.3 and 16.6%), respectively, against MAR 0.428 of *H. pylori* isolates from normal and uterine prolapsed felines. Lastly, the lowest MAR 0.214 from sudden dead felines have sensitivity against the same previous antibiotics plus and imipenem in percent 16.6%.

The highest antibiotic resistance of *H. pylori* isolates was recovered from normal sheep with MAR 1.0 which were tested against all same previous antibiotic discs in the following percents 16.6, 16.6, 16.6, 50, 33.3, 8.3, 50, 66.6, 66.6, 66.6, 83.3, 83.3, 83.3 and 100% but the lowest MAR have sensitivity to all same antibiotics except nalidxic 0%. Resistance isolates of sheep against nalidxic, ciprofloxacin, penicillin and imipenem, cefazoline, gentamycin, cephalothin, amikacin, clarithromycin, metronidazole and rifampicin, levofloxacin, tetracycline and amoxicillin in following percent 100, 83.3, 83.3, 83.3, 66.6, 66.6, 66.6, 50, 8.3, 50, 33.3, 16.6, 16.6 and 16.6%.

Sensitivity of felines and sheep against amoxicillin (100 & 83.3%); 83.3% against tetracycline; (50 & 83.3%) against levofloxacin; 33.3% against rifampicin; 50 % against metronidazole;(33.3 & 83.3 %) against clarithromycin plus (33.3 & 50 %) against amikacin plus (16.6 & 33.3%) against cephalothin, penicillin and gentamycin plus (0 & 33.3%) against imipenem. Intermediate sensitivity (16.6 & 0%) against cefatizidine, in addition to tetracycine and levofloxacin; (16.6 & 33.3%) against rifampicin and 16.6% against cephalothin.

Resistance against gentamycin and cefatizidine was 83.3% & 66.6% but 50 & 66.6% against ciprofloxacin; 50% against amikacin and metronidazole; 100% against nalidxic; (50 & 16.6%) against clarithromycin; (50 & 33.3%) against rifampicin;(33.3 & 16.6%) against levofloxacin and (0 & 16.6%) against amoxicillin and tetracycline but penicillin have resistance (100 & 83.3%) and imipenem with resistance 83.3%.

Table (7) show that antimicrobial resistance to antibiotics such as nalidixic acid, ciprofloxacin, penicillin, imipenem, cefazolin, gentamycin, cephalothin, amikacin, clarithromycin, metronidazole, rifampicin, levofloxacin, amoxicillin and tetracycline are 100 %, 91.7%, 91.7, 83.3%, 75.0%,75%, 83.3%, 50.0%, 33.3%, 50.0%, 41.7%, 25.0 %, 8.3% and 8.3 %, respectively. All *H. pylori* isolates were sensitive against amoxicillin, tetracycline, levofloxacin, clarithromycin and metronidazole and amikacin (91.7, 83.3, 66.7, 58.3, 50, 41.7 and 33.3%,

respectively but cephalothin and gentamycin (25%) and cefazoline was (16.7%).

Figure (1, 2, 3 & 4) shows results of table (1, 2, 3 & 4). All isolates examined by gram staining as gram negative bacilli or coccobacilli in gull winged form as one isolate has represented in **Figure (5).**

Figure (6) shows bands 522bp at lane C +ve and lanes from1 to 12 using ladder 100bp for *16srRNA H. pylori* isolates.

Table (1): *Total H. pylori confirmed by PCR from total urease and nitrate reductive isolates of sero-examined sheep and felines samples in provinces of Upper Egypt.*

Test	+ve	-ve	Total	Serotypes					
				(+)	(-)	(+)	(-)	(+)	(-)
Serology	(106) S:71+ F:35	(29) S:12+ F:17	(135) S:83+ F:52	Hurghada		Luxor		Sohag	
				S:20 F:12	S:4 F:12	S: Nil F: 23	S: Nil F: 5	S: 51 F: Nil	S :8 F: Nil
PCR	7 S:3 + F:4	5 S: 3 + F: 2	12 S:6 + F:6	S: Nil F: Nil	S: 2 F:2	S: Nil F:4	S: Nil F: Nil	S: 3 F: Nil	S:1 F: Nil
%	6.6% S: 4.2 F:11.4	17.2% S: 25 F: 11.7	8.8% S: 7.2 F: 11.5	Nil	S:50 F:16.6	S: Nil F: 17.3	Nil	S: 5.8 F: Nil	S: 1.25 F: Nil
U: N +ve	17:23 1:0.7	19:21 1:1.1	36:44 1:1.2	7:13 (1:1.8)	11:14 1:1.2	5:5 1:1	1:0	5:5 1:1	7:7 1:1
U: N -ve	89:83 1:0.9	10:8 1:0.8	99:91 1:0.9	25:19 (1:0.7)	5:2 1:2.5	18:18 1:1	4:5 1:1.2	46:46 1:1	1:1

U: ureolytic & N: nitritic. T: total. No: number. +ve: positive. -ve: Negative, F: felines and S: Sheep.

Table (2): *Total H. pylori estimated by multiple antibiotic resistance MAR and confirmed by PCR from total urease and nitrate reductive isolates of sero-examined sheep in Hurghada and Sohag.*

Result	Serology		PCR		U: N		MAR
Hurghada							
	No	%	No	%	+ve	-ve	
+ve	20	83.3	0	0%	1:7	19:13 (1:0.6)	Nil
-ve	4	16.7	2	50	2:4 (1:2)	2:0 (1:0)	1.0 0.643
Total	(24)		2	8.3	3:11 (1:3.6)	21:13 (1:0.6)	-
Sohag							
+ve	51	86.4%	3	5.8%	5:5	46:46	0.714- 0.5- 0.07
-ve	8	13.7	1	1.25	7:7	1:1	0.286
Total	(59)		4	6.7	12:12	47:47	-
Sum +ve	71	85.5	3\83	3.6%	6:12 (1:2)	65:59 (1:0.9)	-
-ve	12	14.4	3\83	3.6%	9:6 (1:0.6)	3:1 (1:0.3)	
Total	(83)		6	7.2%	15:18 (1:1.2)	68:60 (1:1.1)	

U: ureolytic & N: nitritic. T: total. No: number. +ve: positive. -ve: Negative.

Table (3): *Total H. pylori estimated by multiple antibiotic resistance MAR and confirmed by PCR from total urease and nitrate reductive isolates of sero-examined felines samples in Hurghada and Luxor.*

Felines	Serology		PCR		U: N		MAR
Hurghada							
Result	No	%	No	%	+ve	-ve	
+ve	12	50%	0	0	6:6	6:6	Nil
-ve	12	50	2	16.6	9:10 (1:1.1)	3:2 (1:0.6)	0.857
Total	(24)		2	8.3	15:16 (1:1.06)	9:8 (1:0.8)	-
Luxor							
+ve	23	82.1%	4	17.3%	5:5	18:18	(0.857) -0.428- 0.214
-ve	5	17.9	0	0	1:0	4:5	Nil
Total	28		4	14.2	6:5	22:23	-
Sum (+ve)	35	67.3%	4\52	7.6%	11:11	24:24	-
-ve	17	32.7	2\52	3.8%	10:10	(7:7)	
Total	(52)		6\52	11.5%	21.:21	(31:31)	

Table (4*): MAR of different biotypes H. pylori isolates from sero-examined felines cases.*

H. pylori **infected felines of totally clinical & non clinical cases**	**Serology**	**Non nitritic**	**Nitritic**	**ureolytic**	**MAR**
1. Constipated (1\3)	-	-	+	+(stool)	0.857
2. Panoleukopenia (1\2)	-	-	+	+(stool)	0.857
3. Diarrheal (1\20)	+	+	-	+(gastric)	0.857
4. Normal (1\4)	+	+	-	+(gastric)	0.428
5. Uterine prolapsed	+	+	-	+(gastric)	0.428
6. Sudden dead	+	+	-	+(gastric)	0.214

Table (5): *MAR of different biotypes H. pylori isolates from sero-examined sheep specimens.*

Sheep Specimens	Serology	Non nitritic	Nitritic	Ureolytic	MAR
1. Normal gastric	-	-	+	-	1.0
2. Normal gastric	-	-	-	+	0.643
3. Congested gastric	+	-	-	+	0.714
4. Congested gastric	+	-	-	+	0.5
5. Congested gastric	+	-	-	+	0.071
6. Milk	-	-	+	+	0.286

Table (6): Total antimicrobial susceptibility test results from *H. pylori* isolates of both groups of felines and sheep

N	N	CP	P	I	CZ	G	CN	AK	Cl	M	R	lev	T	Am
F1	R	R	R	R	R	R	R	R	R	R	R	R	S	S
F2	R	R	R	R	R	R	R	R	R	R	R	R	S	S
F3	R	R	R	R	R	R	R	R	R	R	R	I	S	S
F4	R	R	R	R	R	R	I	I	S	S	S	S	I	S
F5	R	R	R	R	R	R	S	S	I	S	I	S	S	S
F6	R	R	R	I	I	S	I	S	S	S	S	S	S	S
TR	100	100	100	83	83	83	50	50	50	50	50	33	0	0
TI	0	0	0	16	16	16	33	16	16	0	16	16	16	0
TS	0	0	0	16	0	16	16	33	33	50	33	50	83	100
S1	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S2	R	R	R	R	R	R	R	R	S	R	S	S	S	S
S3	R	R	R	R	R	R	R	R	S	R	R	S	S	S
S4	R	R	R	R	R	R	R	S	S	S	I	S	S	S
S5	R	I	I	I	S	S	S	S	S	S	I	S	S	S
S6	R	R	R	R	S	S	S	S	S	S	S	S	S	S
TR	100	83	83	83	66	66	66	50	8.3	50	33	16	16	16
TI	0	16	16	16	0	0	0	0	0	0	33	0	0	0
TS	0	0	0	0	33	33	33	50	83	50	33	83	83	83

R: resistant; I: intermediate and S: sensitive. F: feline. S: Sheep.
TR: Total resistant. TI: Total intermediate. TS: Total sensitivity.
83~ 83.3. 16~16.6. 33~33.3.

Abbreviation of table (6 & 7): (NA): Nalidixic acid; (CP): Ciprofloxacin; (P): Penicillin G; (IPM): Imipenem; (CZ): Cefazoline; (G): Gentamicin; (CN): Cephalothin; (AK): Amikacin; (CL): Clarithromycin; (M): Metronidazole; (RF): Rifampicin; (L): Levofloxacin; (T): Tetracycline; and (AMX): Amoxicillin

Table (7): *Total antimicrobial susceptibility test results from H. pylori isolates of felines and sheep*

T	N	CP	P	I	CZ	G	CN	AK	Cl	M	R	lev	T	Am
R	100	91	91	83	75	75	58	50	33	50	41	25	8	8
I	-	8	8	16	8	-	16	8	8	-	25	8	8	-
S	-	-	-	-	16	25	25	41	58	50	33	66	83	91

91~91.7. 66~66.7. 41~41.7. 16~16.6. 33~33.3. 83~83.3. 8~8.3.

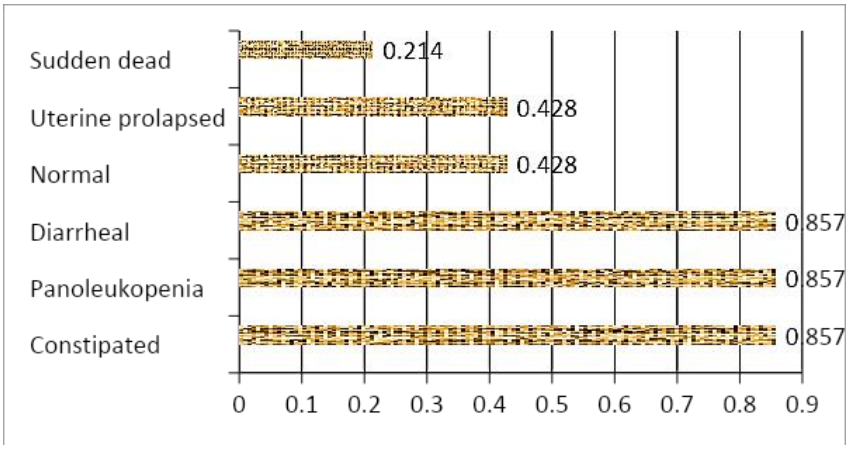

Figure (1): MAR index of *H. pylori* isolates from *H. pylori* infected felines of totally clinical & non clinical cases

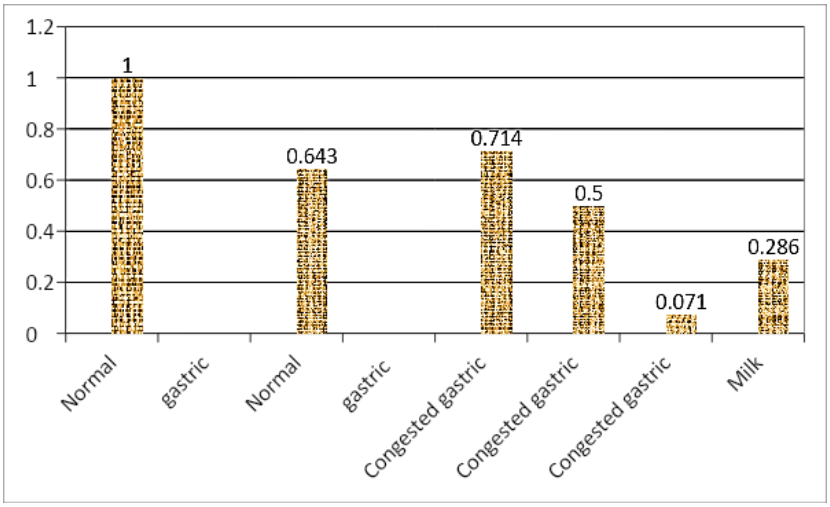

Figure (2): MAR index of *H. pylori* isolates from specimens of sheep

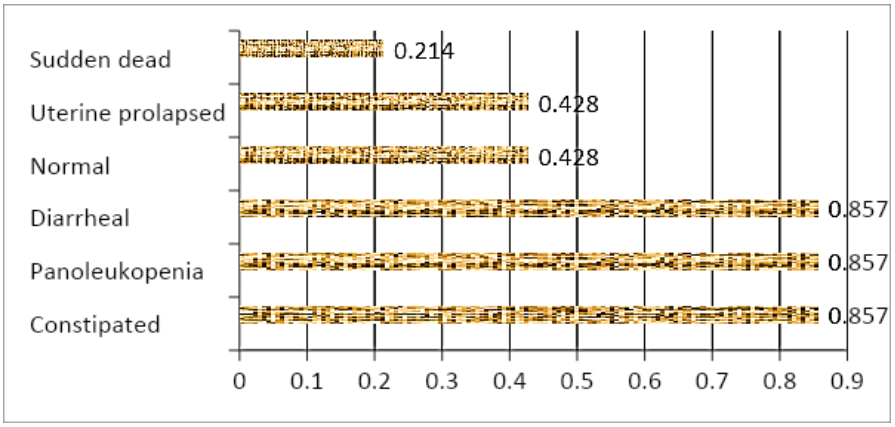

Figure (3): Antibiogram of *H. pylori* isolates Sensitivity

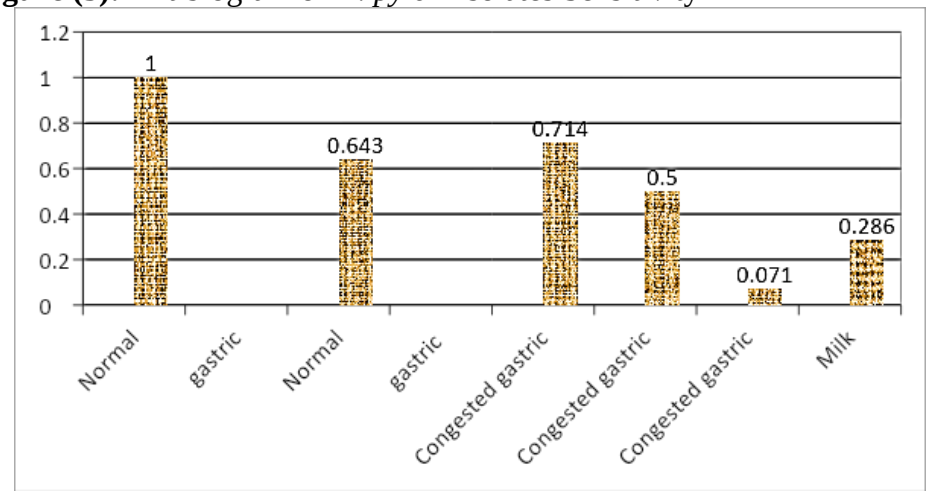

Figure (4): Antibiogram of *H. pylori* isolates Resistance

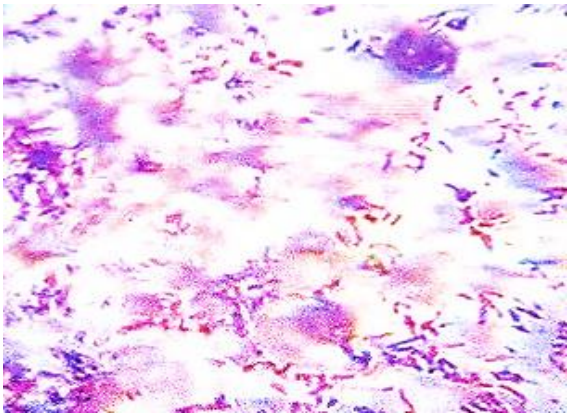

Figure (5): Gram negative gull winged *H. pylori* isolate.

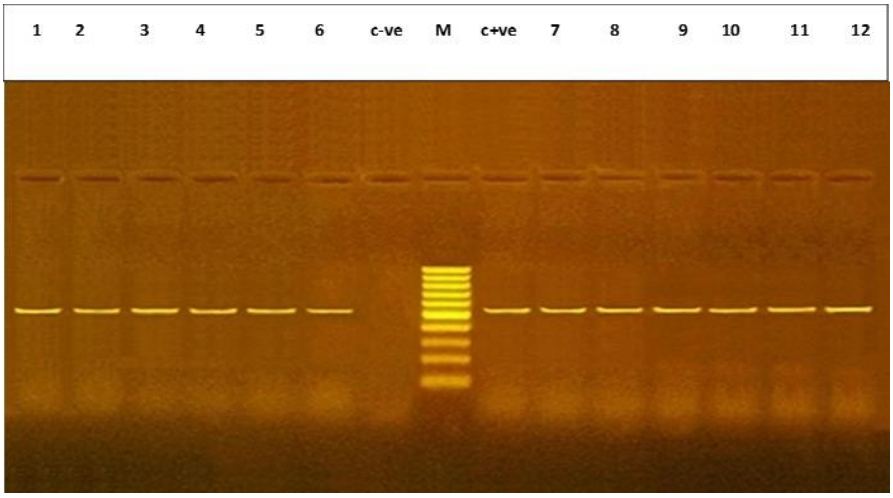

Figure (6): Agarose gel electrophoresis of PCR amplification products using *16srRNA* (522 bp) as specific primer for identification of *Helicobacter pylori*
Lane M: 100bp ladder as molecular DNA marker.
Lane C+ve: Control positive for *16srRNA* of *Helicobacter pylori* ATCC 43504
Lane C-ve: Control negative *E. coli* K12 DH5α.
Lane from 1 to 12: Positive *H. pylori* strains for *16srRNA*

Discussion

Epidemiology of *H. pylori* infection is complicated by both acquisition and loss of infection in different ethnic groups moving from high- to low-income countries ***Fernando et al.* (2001).** To be suggested, Hurghada is the best example for high antibiotic resistance *H. pylori* in socioeconomic status against unprecedented enzymatic nitric oxide NO detoxifying system like microbial protection against nitrosative stress in vivo ***Justino et al.* (2012**), resulted into seronegative felines and sheep (50 & 16.7%) as shown in **table (1**) have the highest multiple antibiotic resistance of *H. pylori* isolates in percent (8.3%) from constipated and pan-leukopenia felines 0.857 and the second multiple antibiotic resistance MAR isolates against normal gastric sheep (0.643) as shown in **table (4 & 5**). Seronegative felines and sheep in Hurghada recovered nearly adequate number of nitrate reduction isolates in proportional to urease activity isolates into U & N ratio (89 & 83) and (10 & 8), respectively as shown in **table (2 & 3**) encouraging role of nitrate on antibiotic resistance followed by excess iron or nickel in soil and diet of host either be felines or sheep, respectively which effect on conversion of U: N isolates ratio across seroprevalence ***Dykhuizen et al.* (1998)** and ***Ansari et al.* (2017)**. Sensitivity against rifampicin or clarithromycin (33.3%) changed upon chelation with host receptor for iron ***Raghu et al.* (1995**) or nickel ***Rowinska-Zyrek et al.*** (**2014**) as attractant or repellant urea,

respectively as rich in soil of Hurghada that alter gene expression (***Haley & Gaddy,* 2015**) or upon slowing rate of ribosomes action according to geographic area ***Kim et al.* (2015)** respectively to inhibit urease activity and nitrogen utilization. Ribosomes of *H. pylori* as nitro reductase encoding *rdxa* gene forming non-toxic metronidazole when bind with nitro product (***Leitsch,* 2019**), to became resistant intermediately (50%) as shown in **table (7)**. Inhibition of urease gene expression for utilization nitrogen inhibit protein synthesis and cell wall of *H. pylori* isolates sensitivity against tetracycline and amoxicillin or binding with topoisomerase *H. pylori* that was inhibited against sensitivity of levofloxacin ***Kim et al.*** (**2015**) in percent (83.3%) as shown in **table (7)** when *16srRNA H. pylori* was being mutated**.** Equality of nitrate reduction and urea utilization isolates ratio were recovered from clinical examined felines of Luxor (0.857, 0.428 & 0.214) and congested gastric seropositive sheep of Sohag with high and lowest MAR (0.071, 0.5 & 0.714) plus milk (0.286), as shown in **table (3 & 4)** where susceptibility antibiotics occur with balanced U\N isolates ratio of other denitrifying bacteria ***Safonov et al.* (2018**) either be totally positive or negative (12 & 12) or (47 & 47), respectively of isolates from seropositive sheep (5 & 5) or (46 & 46) and (7 & 7) or (1 &1) from seronegative sheep as shown in **table (2 & 3)** drive to sensitivity of *Helicobacter* in balanced microbiota normal felines of Luxor and Sohag as rural area**.** Seropositive pet and farm animals in Luxor and Sohag were not recovered nitritic *H. pylori* isolates among U: N isolates ratio (1:0.7) as shown in **table (3)** easily to inhibit urea and not assimilat nitrogen resulted into low resistance of four isolates of sheep in Sohag (33.3 & 50%) against clarithromycin, rifampicin and levofloxacin, respectively that act on ribosomes and *DNA gyrase* ***Mégraud*** (**1998**) but look to be sensitive against tetracycline and amoxicillin (83.3%) as shown in **table (6)** that inhibit penicillin binding protein competitively with B-lactamase antibiotics ***Dore et al.*** **(1999**) to inhibit synthesis cell wall that be homologous and cross react with several bacteria. Low immune felines as seronegative constipated and pan-leukopenia felines (50%) in pet clinics of Hurghada, have nitrate reduction from urea, was more resistant against the almost antibiotics used for eradication isolates of seropositive sheep except metronidazole that its mechanism of resistance 50% depend on binding of ribosomes with nitro product of nitro-reduction isolates that present in seronegative sheep of Hurghada and milk of delivered sheep in Sohag where be more susceptible in percent 83.3 % as shown in **table (3 & 6)** according to transporting un-binding *Iq*A cytoplasmic urease ***Krishnamurthy et al.* (1998**) for

nitrogen assimilation. Less susceptibility to serological response made seronegative felines isolates were resistant against the most same antibiotics of seropositive sheep isolates 83%, 49.8%, 33.3% & 16.6% as shown in **table (6)**. Reduced risk *H. pylori* seropositivity in such as pet clinical cases (76.3%) to recover (11.4%) or few post mortem changes of slaughtered seropositive farm animal (85.5%) were associated with excess iron in such host susceptible cases as felines to isolate (3.6%) as shown in **table (1)** interpretating spontaneous elimination of *H. pylori* infection (i.e., seroversion) from dominant inhabitant of stomach felines only through intraluminal felines pH below 4.0, or through mis regulating fur genes by expressing low CD4 count from parietal cells of an animal model (***Xia and Talley*, 1997)** that decrease viability of *H. pylori* and efficacy of dose antibiotic therapy. According to weak and strong urease gene expression, all seronegative farm animals and pet as felines in Luxor have *H. pylori* isolates with no utilization urea in proportional to nitrate producing in percent (14.4%) & (17.9%), respectively as shown in **table (1),** were recovered in few number among more U:N isolates ratio (1:1) than U: N isolates ratio (1: ∞) from seropositive felines as shown in **table (3),** explain why higher percent in urban area of seronegative stool antigen of felines cases like pet clinics of Hurghada and pet hospitals of Luxor 23.7% was more than percent of negative *Ig*G 13.7% from seronegative sheep in rural area (Sohag) to recover felines isolates in percent 3.8% than percent of sheep isolates 1.25% as shown in **table (3).** Similar to U: N isolates ratio (1:1.8) that isolated from all seropositive pet (17.3) and farm animals (5.8%) in Sohag were more than U\N -ve isolates ratio (1:1.2) as shown in **table (1)** especially sheep have U: N isolates ratio (1:3.6) as shown in **table (2)** to evidence that gastric tissue of sheep in rural area as Sohag can act as a reservoir to *H. Pylori* and disseminate the pathogen in feces and milk then transmitted to human during uses of unpasteurized milk, or meat ***Kareem and Al-Maaly*, (2021)**. Seronegative sheep isolates closed to worker of sheep abattoir was also in higher percent 25% against seropositive sheep in percent 3.6% as shown in **table (3)** due to metal-regulated urea diet fasting sheep on digesting low protein diet with less utilization nickel-based nickel dehydrogenase urea ***Sarraseca et al.* (1998).** The highest percent isolation *H. pylori* in percent 50% from seronegative sheep (16.7%) from bad hygienic area as abattoir of Hurghada (urban area) as shown in **table (1)** was dissimilar to result of ***Kareem and Al-Maaly*, (2021)**, the largest percent of *H. pylori* isolation occur in people with bad socio-economic habitat or rural area as AL-Rumetha with percentage 15%, comparing between

rural and city of AL-Muthana provinces that slaughter house need long incubation period to reach 9%, resulted into limitation of the risk of cross-reactions with other bacteria that affected by ("sham-feeding") which demonstrated by Pavlov, slaughtering of fasting animals for 24 hours in bad hygienic area made food related neuro activated to hungry animal of appetizing food ***Konturek et al.*** **(2006).** Through the previous findings**,** elimination rate of *H. pylori* either be antibiotic susceptibility or resistance antibiotics which return to host susceptibility to metal regulated urea utilization or cross reaction with other ureolytic or denitrifying bacteria followed by seroprevalence or may return to constructed genomic loci in microbes, according to conversion of non-producing nitrate to utilization urea ratio isolates into nitrate producing from utilization urea isolates occur reversely in between seropositive and seronegative felines of Luxor and Hurghada with different multiple antibiotic resistance MAR against clinical cases especially seronegative clinical examined felines with highest resistance (0.857) as shown in **table (3)**, and with highest multiple antibiotic resistance MAR against normal seronegative sheep (1.0 & 0.643) as shown in **table (2)**. Highest isolation percent in Luxor is 17.3% as shown in **table (1)** from seropositive felines (82.1%) with isolation 14.2% in Luxor (urban area) in propotional to 7.6% as whole isolation from seropositive felines as shown in **table (3)** perhaps return to similar findings of ***Fernando et al.*** **(2001**) as *Ascaris* infection and a history of *Mycobacterium bovis* BCG immunization that increasing trans-exudation of serum components as weak urease activity (HP-W) and strong urease activity (HP-S) as *H. pylori* HP0013 ***Sarraseca et al.*** **(1998)** for colonization against sensitivity to antibiotics (***Leitsch,*** **2019).** An increased risk of *H. pylori* acquisition in urban occur when nearly equilibrium between isolation *H. pylori* from pet clinics of Hurghada in percent isolation 16.6 % as pathogens from sero-negative constipated and pan-leukopenia felines, and isolation *H. pylori* as commensals from apparently healthy felines with more highly seropositive closed to technicians of pet hospitals in Luxor 17.3 % as shown in **table (2),** may because an interaction between *Helicobacter* and viral or parasitic infections modifying the outcome of infective processes and causing lower CD+ cells against other viral infection and immune-defficiency ***Fernando et al.*** **(2001**) resulted into failure antibiotic therapy**.** Sohag recovered *H. pylori* isolates (6.7%) especially of seropositive sheep (86.4%) had higher *H. pylori* isolates in percent 5.8 % closed to meat and milk consumers from sheep market than the lowest isolation in percent 0 % of seropositive sheep (83.3%) from

abattoir of Hurghada as urban area, as shown in **table (1),** is similar to *H. pylori* in abomasums tissue of adult sheep in rural area which set than lamb of urban area (***Kareem and Al-Maaly,* 2021).** Rural area which spread between slaughters or consumers in abattoir and market of Hurghada and Sohag or from infection environment around owner of felines in pet hospital Luxor may be acquired mutant resistant gene microbial. Sheep is nearly sensitive to metal regulation of *H. pylori* urea utilization ***Bury-Moné et al.* (2004)** for control mechanism of antibiotics on isolates upon disease severity according to expression the downstream urease genes ***Fernando et al.* (2001**) based upon changes in titers of specified amounts of urease expression (***Follmer,* 2010)** upon iron chelation with metal ions in water and diet of geographical region where normal host as PH of gut ruminal sheep (***Lea and Maija,* 1974)** was in response to any amount of urea **(*Panday*, 2011)** by repellant urea as excess nickel in diet ***Sanders et al,* (2013**). Common antibiotics as tetracycline and amoxicillin (91.7% & 83.3%) against seropositive sheep was being used in Luxor, s hospitals seropositive felines such as diarrheal felines where have no nitrate reduction with moderate susceptibility against clarithromycin, rifampicin and levofloxacin, respectively 50% and lastly became more resistant to the most antibiotics 16.6%, 33.3% & 50% against seropositive normal, sudden dead and uterine prolapsed felines as shown in **table (3 & 6**). Perhaps due to some occupational groups contact with host susceptibility as veterinarian in pet clinicians or abattoir of urban area such as Hurghada may pose an additional risk of infection that be resistant against most antibiotics. That antimicrobial resistance to antibiotics such as nalidixic acid, cephalothin, penicillin, imipenem, cefazolin, tetracycline, gentamycin, ciprofloxacin, amikacin, metronidazole, rifampicin, clarithromycin, levofloxacin, and amoxicillin are 100 %, 91,7%, 83,3%, 75.0 %, 8.3 %, 75.0 %, 58.3 %, 50.0 %, 50.0 %, 41,7%, 33.3 %, 25.0 %, and 8.3 %, respectively, as shown in **table (7)** in similar results of study (***Ali,* 2016**) which reported low resistance from gastric poultry of Assiut governorate against nalidixic acid and penicillin. Dissimilar to the World Health Organization (WHO) report, the rate of resistance to metronidazole ranged 20–38% but resistance against clarithromycin 14–34% where suggested that the therapeutic regimens with less than 80% efficacy are considered as treatment failure (***Keikha & Karbalaei,* 2021**). Resulted into host susceptibility against un-balanced nitrate product to urea utilization where higher isolation from seronegative sheep and felines 25% & 11.7%, respectively was shown than 4.2% & 11.4% from seropositive animals, respectively as present in **table (3)**

although seroprevalence of animals was 85.5% & 67.3%, respectively as shown in **table (1)** & **figure (4)** in total twelve isolates**.** Similar to (***Faten et al,* 2018)** who revealed no isolation from positive *H. pylori* seroprevalence felines (33.3%), compared to seroprevalence of humans (90%) ***Alboraie, et al.* (2019).** Breeding on soil and pipes water especially in autumn season of urban area as Hurghada changes metal regulation of urea transporting by nickel in sheep or iron in felines according to low gastric PH under 4 as in felines to be more suitable for normal habitat *H. pylori* ***Mobley et al.* (2001**) than sheep have PH above 5 to be protective resulted into higher isolation from gastric and stool of felines 11.5% than sheep 7.2% as shown in **table (3)**, similar to the most commonly detected genotypes *16s RNA* in the liver and bile samples of felines (24.3%**)** ***Sacha et al.* (2004)** which is higher than isolation from slaughterhouses (20.67%) (***Kareem and Al-Maaly,* 2021).** To be recommended when nickel is repellant for urea that may be founded in rural area otherwise diet represented usually iron as attractant for urea in less amount in urban area, that evaluation of antibiotic susceptibility test in comparing to biotypes of *H. pylori* may be more diagnostics for effect of antibiotic resistance than serological identification**.** All *H. pylori* isolates were sensitive against amoxicillin, gentamycin, tetracycline, levofloxacin, rifampicin and metronidazole was (91.7, 83.3, 66.7, 58.3, 50, 41.7 and 33.3%, respectively and cephalothin 16.7, amikacin and ciprofloxacin (25%) as shown in **table (7).** Amount of nitrogen to urea utilized presented in Hurghada then Luxor and Sohag will be future point research on epidemiological prevalence of *H. pylori* surveying the best identification through antibiotic susceptibility than culture or serology spending time, cost and effort, preferring quantitative PCR for determination rate of elimination or rate of acquisition against other flora which need the right antibiotic treatment.

Recommendation: Determination of nitrate product in soil and biological samples will be more preferable step for programming eradication *H. pylori* by antibiotics as Hurghada to be choosing by crisper sequencing of multiple antibiotic resistance gene region on suspected *H. pylori* infected vet clinical cases in the future researches, in addition to notification veterinarians from misusing antibiotics in area have balanced utilization urea to nitrogen assimilation as Sohag and Luxor provinces.

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الملخص العربي

المقاومة للأنماط البيوكيميائية لعزلات الجرثومه الحلزونيه للمضادات الحيوية المتعددة فى الأغنام والقطط المختبره سيرولوجيا في صعيد مصر

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1.قسم الميكروبيولوجى بكليه الطب البيطرى جامعه قناه السويس. 2. قسم الأمراض المشتركه بكليه الطب البيطرى جامعه قناه السوس. 3. قسم الميكروبيولوجى بكليه الطب البيطرى جامعه سوهاج. 4.قسم الأمراض المشتركه بكليه الطب البيطرى جامعه الوادى الجديد. 5. طالب دكتوراه ميكروبيولوجى بكليه الطب البيطرى جامعه قناه السويس.

الملخص العربي

أظهرت هذه الدراسة المقاومة للمضادات الحيوية المتعدده لأنواع بيوكيميائية مختلفة من عزلات الجرثومه الحلزونيه من القطط والأغنام في صعيد مصر.من إجمالي مائة وخمسة وثلاثين عزلة من (6 عينات نسيج معدة و46 عينه براز)من 52قطه و(66 عينه نسيج معدة و 17عينه لبن)من 83 غنم في محافظات الغردقة والأقصر وسوهاج.تم فحصهم سيرولوجيا باستخدام اختبار مستضد انتجين البرازللقطط و اختبار التلزن للأغنام في إجمالي 67.3%من اجل زرعها في أنابيب معقمة 5 مل من مرق ثيوغليكولات مضاف اليها 10 ميكرولتر من الهيمين لعينات القطط أو غيرمضاف لعينات الاغنام فى نسبه اكسجين محدوده عند 37 درجة مئوي خلال 36 ساعة ، و يعاد زراعتها على أطباق مملوءة من 500 مل من أجار القلب والدماغ الدموي المضاف إليه 135 ميكرولتر من محلول اليوريا 40٪، نوفوبيوسين 500 ميكرولتر، فانكومايسين 25 ميكرولتر، وأمفوتيريسين ب 350 ميكرولتر لمدة 5 أيام وتم فحصها مجهريا على صبغة سالبة الجرام، وكيميائيا على الأكسيداز الموجب، والنشاط الأنزيمي المتغير لاختزال اليورياز والنترات الذي تم تأكيد عن. بوليمير16اوكسيدوريبونيوكليي أربع عزلات في كل محافظة بنسبة إجمالية 8.8 ٪. خلال تفاعلسلسلة لتسجيل أعلى مقاومة للمضادات الحيوية المتعددةعن طريق اختبار الحساسية للمضادات الحيويه (1.0)و (0.643) و (0.857) بين نسبة واسعة من عزلات اليورياز وعزلات اختزال النترات (1:1.8) فى الغردقة. لكن النسبة المتساوية منها تضمنت أقل معدل مقاومة للمضادات الحيوية المتعددة (0.07) بالإضافة إلى (0.5) و (0.286) و (0.714) فى سوهاج.