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Insight of Molecular Prevalence on Antibiotic Sensitive *H. pylori* Biotypes from Apparently Healthy and Clinical Illness Felines and Sheep

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Abstract: *Helicobacter pylori* is gram-negative bacteria may transmit through human food chain causing serious health problems in humans worldwide upon misusing antibiotic therapy for animals. Aim of the study represent the incidence of variants biotypes *H. pylori* susceptible to antibiotic in apparently healthy or clinical illness felines and sheep through amplification of *16srRNA*. Four stomachs of 3 apparently healthy and diarrheal feline and two stool of constipated and pan-leukopenia felines, in addition to five gastric sheep from 2 normal and 3 congested plus one milk, selected from 52 felines and 83 sheep, respectively based on traditional cultivation and biochemical differentiation in total twelve *H. pylori* isolates that confirmed by amplification of *16srRNA*, not being recognized by v3-v4 primer as nitrate gram negative bacteria. *H. pylori* isolates were grouped upon urease and nitrate reduction reaction in total percent 50% for each of weak and strong urease biotypes, including 33.3% & 66.6% for (+ve) or (-ve) nitrate reductive biotypes, respectively. Sensitivity of these biotypes was determined against fourteen antibiotic discs by antimicrobial susceptibility test to find highest sensitivity non-producing nitrate biotypes from felines is (87.5%), lesser than sheep (100%) but almost positive nitrate reductive isolates is less susceptible in percent 25%. Moderate sensitivity of weak urease biotypes represents 50% against amikacin, opposite to strong urease isolates (33.3%). Highest sensitivity strong urease biotypes show 83.3% against clarithromycin and levofloxacin, compared to weak urease biotypes 50 & 66.6%, respectively. Bio-typing *H. pylori* is preferable for programming eradication in molecular surveying normal or clinical illness animals.

Keywords: Antimicrobial Susceptibility Test (AST), *H. pylori*, Nitrate, Urease, *16srRNA*, Sheep and Felines

1. Introduction

Helicobacter pylori is slowly growing microaerophilic bacteria strain, has variations supported by Sharma on basis of enzyme profiles [1] included nitro sating species [2] making nitro reductase and nitro reductase function [3]. *H. pylori* representing an unproved zoonotic pathway by cats, dogs, and sheep to humans [4]. Since, felines and sheep were small animal model for gastritis human [5] at an acidic PH below 5, from 1 to 2 in felines [6] and at a neutralized highly acidic PH upon 5 by urea diet in rumen [7], respectively that increased markedly at low PH [8] when identified by minimum inhibitory concentration MICs of antibiotics. After antimicrobial therapy, acidified nitrate can be reduced to nitrite forms ammonia that determined using Mongolian gerbils resulted in high antibiotic resistance in vivo

presenting against unprecedented enzymatic nitric oxide NO detoxifying system for microbial protection against nitrosative stress [9] that play an important role in gastrointestinal mucosal protection, but excess nitrite in gastric lumen play role in *H. pylori* related abnormalities [10] forming N-nitroso compounds by laboratory animal species which is closely related to gastric cancer [11], may cause severe malignant lesions through an oncogenic transformation in transgenic animals [12, 13]. Nitrate reduction was identified biochemically [14] and evaluated genotypically by *16srRNA* of other than *H. pylori* as v3-v4 region coding for nitrate gram negative bacteria [3] and *16srRNA* of conserved region of *H. pylori* [15] for follow up of eradication therapy methods. Elimination rate of *H. pylori* either be antibiotic susceptibility which return to constructed genomic loci in microbes or resistance antibiotics that may return to host susceptibility as clinical cases where Germany involved the first investigation of gut microbiota changes of Mongolian gerbils after 14 months of *H. pylori* infection [16]. Using the Epsilometer (E-test) as concluded as a quantitive disc diffusion antibiotic susceptibility testing method compared to qualitative disc diffusion method [17], which be economic, and the same accuracy [18]. To gain therapeutic and economic targets, evaluation of the nitrate-nitrite-NO pathway that making endocytosis of survival and transmissible *H. pylori* through its outer membrane proteins, play an important regulatory mechanism in pathologic and clinical conditions [19]. Colonization *H. pylori* against sensitivity to antibiotics occurs through long incubation, acquiring urease activity from neighboring bacteria, which were investigated by the urease test [20]. In vivo, a subpopulation of *H. pylori* contains cytoplasmic urease only when *H. pylori* survives in acidic environment where abundant quantities of urease were producing toxic ammonium but suitable amount urease hydrolyses into ammonia and carbon dioxide C02 when urea result in generation of a PH neutral micro-environment [21]. Aim of the present work is determination the best choice antibiotic by antibiotic susceptibility test associated with bio typing *H. pylori* detected by *16srRNA,* recovered from gastrointestinal specimens of apparently healthy and clinical illness felines and sheep that confirmed by amplification of v3-v4 region of *H. pylori* or other than *H. pylori*.

2. Materials & Methods

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

2.1. Sampling

During summer in 2017 to spring of 2021, (6 stomach and 46 stool) of 52 felines and (66 gastric and 17 milk) of 83 sheep were collected. A total number of (6) felines gastric tissues from immediately dead or euthanized cats by intracardiac injection of syringe 3 ml saline [22] at Animal care hospital (ACE) and Blue moon clinician as branch of Animal Friendship Social Organization in Egypt. Seventy-two gastric sample from slaughtered apparently healthy sheep and felines was scrubbed from the inner mucosa of gastric tissue, cut small part to put it into transport media as saline or glycerol until arrival for laboratory [23], and labelled by date, number and type of species. Fourty-six stool swab was directly collected from the rectum with sterile stick or cotton swab into sterile tube with buffer as instructed for stool antigen test and labelled by date, number and sign of illness [24]. Gastric and stool swabs were immersed deeply in buffer or phosphate buffer saline PBS or Thioglycolate (Thio) broth, then enriched in (Thioglycolate broth or brain heart infusion BHI broth with no antibiotics) for inoculating onto plate [25]. The second flow of seventeen milk samples was transported into sterile cups and labelled by date, number and type of species, then preserved in an icebox with refrigerants to transport to the laboratory for centrifugation [26].

2.2. Isolation

Under aseptic condition, enriched samples were inoculated for 36 – 48 hours onto thioglycolate broth (Himedia) India) supplemented with urea [27] and haemin [14] (chlorid) (Roth.co.) Australia) or non-supplemented BHI broth (Oxoid) UK, for sheep isolates under microaerophilic conditions using CampyGen gas kit (10% co2, 85%N2) (Oxoid) (CN 0035A) inside Jar 3.5 liter or 2.5 liter then subcultured for 5 days at 37c on BHI agar supplemented with antibiotics (vancomycin (EMC. UK.), and amphotericin B (Astellas Pharma. US) [28].

2.3. Biochemical Differentiation

One hundred and thirty- five isolates out of totally collected samples were differentiated biochemically [29] by oxidase (Oxoid), catalase reagents, urease media (HKM) Guangdong Huankai, and nitrate reduction media (Peptone) (HiMedia). India [30], using KNo3 reagent composed of (Potassium Nitrate (Nasco). US in weight 0.2 g dissolved into 1 ml distal water D. W) then characterized by gram staining (Himedia) [31] into gram negative rods or coccus. 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, 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.

2.4. DNA Extraction of H. pylori

Using materials of DNA extraction from isolates by (QIa amp Kit) [32].

2.5. Polymerase Chain Reaction (PCR) Detection of 16srRNA H. pylori

Gene Jet Genomic used for DNA purification [32], including 50 µL of PCR Master Mix (EzWay, Koma Biotech, Seoul, Korea), composed of (5uL 10ǿ buffer + MgCL2, 2mMdNTP, 2 uni Taq DNA polymerase) contained 100 ng of the extracted DNA and 25 pm of primer forward (F5 GCGCAATCAGCGTCAGGTAATG3) and reverse (R5 GCGCAATCAGCGTCAGGTAATG3) [33] for amplification in thermal cycler (Eppendorf, Hamburg, Germany) at PCR conditions [34] 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 1Tris\Borate\EDTA (TBE) buffer stained with ethidium bromide to be evaluated on an ultraviolet (UV) transilluminator. The *16srRNA* gene PCR gene product was (522) base pair (bp) and all data were examined using Ladder 100bp [35] (Fermentas).

2.6. PCR Detection of 16srRNA v3-v4 Region

For confirmation of presence of nitrate gram negative other than *H. pylori* using primer 338 F (5ACTCCTACGGGAGCCAGCAG-3) and 806R (5-CGACTACHVGGGTWTCTAAT-3) according to methods of [36].

2.7. Antimicrobial Susceptibility Test (AST)

According to the guidelines stipulated by *"NCCLS" National Committee for Clinical Laboratory Standards for susceptibility antimicrobial standards performance* [2001], 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 [38]. Moreover, the plate was incubated at suitable temperature 25°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 1 for determination of multiple antibiotic resistance (MAR) index by using the formula MAR = Number (No.) of resistance \Total No. of tested antibiotics [39] where isolates classified as intermediate were considered sensitive for MAR index.

Statistical analysis

Principle component and cluster analysis methods were used for antimicrobial susceptibility of *H. pylori* isolates into groups in statistical software suite SAS software ver. 9.2, SAS Institute 2008 [40].

Table 1. *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
Levofloxacin	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

3. Results

Twelve *H. pylori* biotypes were detected by *16srRNA* amplification from 12 (100%) positive and 9 (75%) negative isolates of urease and nitrate reduction that differentiated from 50 positive oxidase isolates among 70 isolates identified by gram negative stain. All *H. pylori* isolates were not detected by amplification of *16srRNA* v3-v4 region as nitrate reducing gram negative bacteria that collected from 6 gastric and 46 stool of felines in addition to 66 gastric and 17 milk samples of sheep in total 135 samples recovered 12 (8.8%) *H. pylori* isolates, given 6 and 21 plus 31 and 10 gram-negative isolates, respectively in total 70 isolates from 179 gram stained and given 6 and 15 plus 22 and 7 oxidase positive isolates, respectively in total 50 isolates from 74 oxidase tested.

Urease and nitrate reduction positive isolates were selected into (2 & 0) from gastric felines, (5 & 5) from stool felines, (15 & 12) from gastric sheep and (3 & 5) from milk sheep to detect *H. pylori* isolates (4, 2, 5 & 1), respectively. Urease and nitrate reduction negative isolates were selected into (4 & 6) from gastric felines, (10 & 10) from stool felines, (7 & 10) from gastric sheep and (4 & 2) from milk sheep to detect

H. pylori isolates (4, 0, 5 & 0), respectively.

Table 2. *Molecular prevalence of biotypes H. pylori isolates from collected isolates of felines and sheep specimens.*

Animal specimens (No. (135)	Biochemical differentiation of isolates (179)				Bio typing of gram-negative positive oxidase isolates (50)				Molecular detection of (+ve\-ve) (12) biotypes			
	Gram stain		Oxidase		Urease		Nitrate reduction		+ve V3-v4	-ve	+ve *16srRNA*	-ve
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
Gastric felines (6)	6	6	6	0	2	4	0	6	0	0	4	4
Gastric sheep (66)	58	33	22	11	15	7	12	10	0	0	5	5
Stool felines (46)	35	21	15	10	5	10	5	10	0	0	2	0
Milk sheep (17)	10	10	7	3	3	4	5	2	0	0	1	0
Total	109	70	50	24	25	25	22	28	0	0	12	9

3.1. Gram Stain of Representative Isolate

All isolates examined by gram staining as gram negative bacilli or coccobacilli in gull winged form as one isolate has represented in Figure 1.

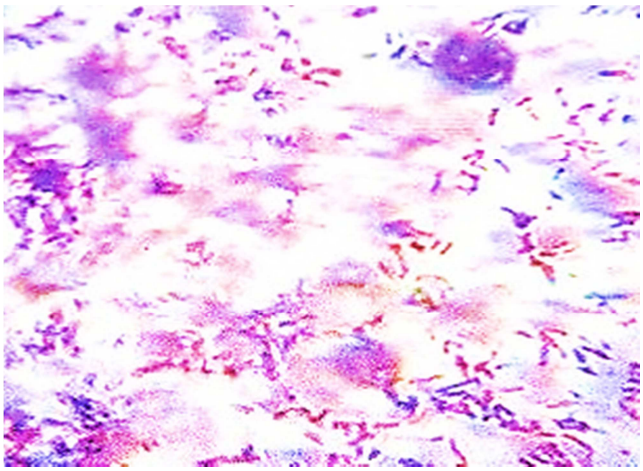

Figure 1. *Gram negative gull winged H. pylori isolate.*

3.2. PCR Amplification of 16srRNA H. pylori

Figure 2 shows bands 522bp at lane C +ve and lanes from1 to 12 using ladder 100bp for *16srRNA H. pylori* isolates.

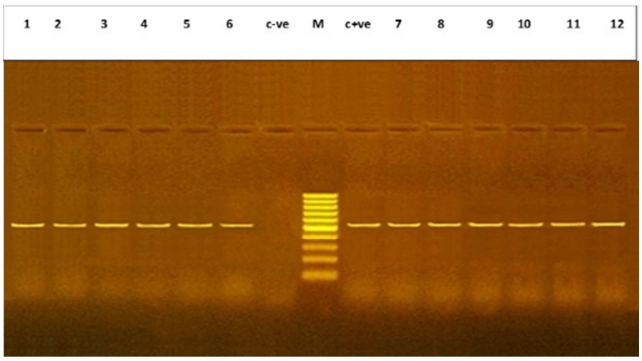

Figure 2. *Agarose gel electrophoresis of PCR amplification products using 16srRNA (522 bp) as specific primer for identification of Helicobacter pylori.*

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.*

3.3. Antimicrobial Sensitivity

All *H. pylori* isolates were sensitive against amoxicillin, tetracycline, levofloxacin, rifampicin, clarithromycin, metronidazole and amikacin (91.7, 83.3, 66.7, 33.3%, 58.3, 50, 41.7), respectively and cephazolin (16.7), gentamycin and ciprofloxacin (25%).

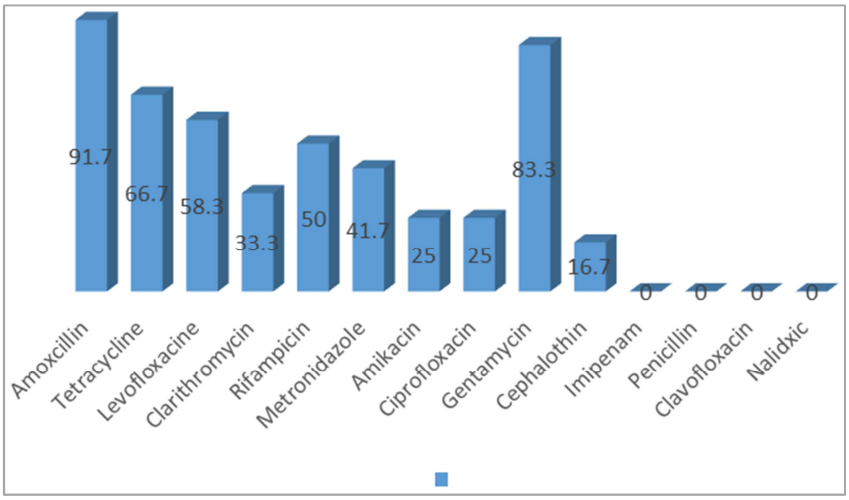

Figure 3. *Antibiogram of H. pylori isolates sensitivity.*

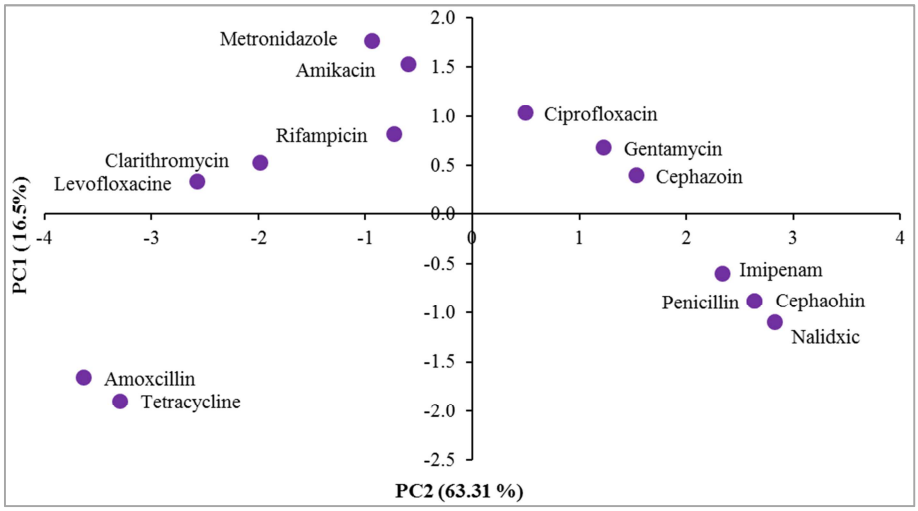

Figure 4. *Principal component analysis of susceptibility antibiotics against H. pylori isolates.*

3.4. Bio-typing of 16srRNA H. pylori Isolates and Antibiotic Sensitivity

Table 3 show six weak urease *H. pylori* isolates in percent 50% were recovered from 4 gastric of three apparently healthy & diarrheal felines, and one stool of pan-leukopenia feline, and one gastric normal sheep from total twelve *H. pylori* isolates confirmed by *16srRNA* PCR as shown in figure 2 in similar to the same percent 50% of six strong urease *H. pylori* isolates that recovered from one stool of constipated feline, and two gastric of each normal and gastritis sheep plus milk sample, to be susceptible against tetracycline, amoxicillin and levofloxacin as the 3rd susceptible antibiotics in percent 83.3% with exception sensitivity of levofloxacin in percent 66.6% against four of weak urease felines isolates that be the highest sensitive drug combined with tetracycline and amoxicillin in 100% percent especially when were tested against eight non reductive nitrate isolates from total isolates in percent 66.6% which recovered from 3 & 2 normal of felines and sheep, respectively and from diarrheal feline and two gastritis sheep that become moderate sensitive in percent 50% with exception of sensitivity of levofloxacin was 25% against two illness felines (constipated and pan-leukopenia) and one milk sample from total four nitrate reduction isolates in percent 33.3% from total isolates including one isolate from normal sheep. Clarithromycin and rifampicin were the second and fourth susceptible antibiotic in percent 87.5% & 75%, respectively against almost 8 non-reductive nitrate isolates (7 & 6) isolates, respectively. Followed by descending manner (83.3%, 50% & 25%) and (66.6%, 66.6% & 25%) as the fifth susceptible antibiotic against strong and weak urease then nitrate reductive isolates. Metronidazole and amikacin represent the sixth susceptible antibiotic in percent 62.5% against non -reductive nitrate isolates to be moderate sensitive as seventh susceptible antibiotic in percent 50% against weak and strong urease isolates but amikacin have 33.3% against strong urease isolates in similar to ciprofloxacin then both antibiotics and ciprofloxacin were descending to 25% against nitrate reduction isolates. Both Gentamycin and cefazolin have higher sensitivity against strong urease isolates in percent (33.3%), respectively plus imipenem sensitivity 16.6% then descend to 25% against nitrate isolates either be reductive and non-reductive till to be resistant against weak urease isolates. Conversely to penicillin and cephalothin which have higher sensitivity against strong urease isolates in percent 16.6% that descend to 12.5% against non-reductive nitrate isolates till to be resistant against weak urease and nitrate reductive isolates as resistance of nalidixic against total twelve *H. pylori* isolates in percent 100%. All *H. pylori* isolates were not amplificated by v3-v4 primer coding of nitrate gram negative bacteria.

3.5. Biochemical Reaction and Antibiotic Susceptibility to Felines and Sheep

Table 4 and Table 5 show sensitivity of three normal felines and two sheep plus one diarrheal feline and two gastritis sheep form eight non-reductive nitrate isolates as 5 weak urease isolates from (4 felines & one normal sheep) and five strong urease isolates from 1 normal plus 2 gastritis sheep in addition to isolates of (milk) and one gastritis sheep, as nitrate reductive isolates were sensitive in descending manner against tetracycline, amoxicillin and levofloxacin that be resistant against two isolates of pan-leukopenia and constipated felines as nitrate reductive weak and strong urease, respectively.

All non- reductive nitrate isolates of four felines were sensitive against metronidazole and amikacin, in addition to rifampicin and clarithromycin, except one isolate from one gastritis sheep in addition to one diarrheal feline as strong and weak urease, respectively plus nitrate reductive weak urease isolate of pan-leukopenia felines were resisting otherwise other strong urease nitrate reductive isolate of milk is sensitive but one strong urease non-reductive nitrate isolate of one normal and two gastritis sheep resist metronidazole, amikacin and ciprofloxacin.

One of strong urease reductive nitrate isolates as milk sample plus one weak urease non-reductive isolate from

prolapsed uterine feline were sensitive against gentamycin, cefazolin and imipenem plus other strong urease non-reductive isolate was recovered from one gastritis sheep that be sensitive to almost antibiotics in the present study except nalidixic that be resistant to whole recovered isolates from felines and sheep either be normal or clinical.

Fourteen antibiotic discs used for antimicrobial susceptibility named with those abbreviations as following: (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 3. *Percent of susceptible weak, strong urease, nitrate reduction and non-reductive nitrate of H. pylori isolates against antimicrobial discs.*

Antimicrobial discs	Weak urease *H. pylori* isolates (6\12) 50%	Strong urease *H. pylori* isolates (6\12) 50%	Non- reductive nitrate *H. pylori* isolates (8\12) 66.6%	Nitrate reduction *H. pylori* isolates (4\12) 66.6%	Total sensitivity
(NA)	0%	0%	0%	0%	0%
(CP)	0%	1(16.6%)	1(12.5%)	0%	0%
(P)	0%	1(16.6%)	1(12.5%)	0	0%
(IPM)	16.6%	2(33.3%)	(2)25%	(1)25%	0%
CZ	16.6%	(2) 33.3%	(2) 25%	(1) 25%	16.7%
G	16.6%	(2) 33.3%	(2) 25%	(1) 25%	25%
(CIP)	3(50%)	(2) 33.3%	(4) 50%	(1) 25%	25%
Ak	3(50%)	(5) 83.3%	(5) 62.5%	(1) 25%	41.7%
Cl	3(50%)	(3) 50%	(7) 87.5%	(1) 25%	58.3%
M	3(50%)	(3) 50%	(5) 62.5%	(1) 25%	50%
R	4(66.6%)	4(66.6%)	6(75%)	1(25%)	33.3%
(L)	4(66.6%)	5(83.3%)	8(100%)	1(25%)	66.7%
(T)	5(83.3%)	6(100%)	8(100%)	(3)75%	83.3%
(AMX)	5 (83.3%)	6(100%)	8(100%)	(3)75%	91.7%

Table 4. *Antibiotic susceptibility against variable reaction of urease and nitrate reduction of H. pylori isolates recovered from normal and illness felines.*

Apparently healthy feline cases	MAR	N	U	Susceptible antibiotics	Clinical illness felines	MAR	N	U	Susceptible antibiotics
Normal	0.428	-ve	-ve	T, Amx, Lev, R, M, Cl, AK & Cip	Constipation	0.857	+ve	+ve	T, Amx
Dead suddenly	0.428	-ve	-ve	T, Amx, Lev, R, M, Cl,	Panleukopenia	0.857	+ve	-ve	T, Amx & Lev
Prolapsed uterine	0.214	-ve	-ve	AK, Cip, G, CZ & I	Diarrhea	0.857	-ve	-ve	

U: ureolytic & N: nitritic. (+ve): Strong. (-ve): weak.

Table 5. *Antibiotic sensitivity against variable reaction of urease and nitrate reduction of H. pylori isolates recovered from normal and illness sheep.*

MAR of apparently healthy sheep	N	U	Susceptibility antibiotics
(1). Normal Sheep (1.0)	+	-ve	-
(2). Normal Sheep (0.643)	-ve	+ve	T, Amx, Lev, R & Cl
(3). Normal sheep (0.714)	-ve	+ve	T, Amx, Lev & Cl
(1). Inflammation GIT (0.5)	-ve	+ve	T, Amx, Lev, Cl, M, R & AK
(2). Inflammation GIT (0.071)	-ve	+ve	T, Amx, Lev, R, M, Cl, AK, Cip, G, CZ, I, P & CP
(1). Milk of delivered sheep (0. 286)	+ve	+ve	T, Amx, Lev, R, M, Cl, AK, Cip, G, CZ & I

4. Discussion

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 [41]. All *H. pylori* isolates from felines and sheep that detected by *16srRNA* in percent 8.8% as shown in results of table 2 were sensitive against amoxicillin, tetracycline, levofloxacin, rifampicin, clarithromycin, metronidazole and amikacin (91.7, 83.3, 66.7, 33.3%, 58.3, 50, 41.7), respectively and cephazolin (16.7), gentamycin and ciprofloxacin (25%) as shown in table 3 and figure 3. 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 [42]. For avoiding acquisition of antibiotic resistance from normal host, determination of urea reaction either be weak or strong is selective step for administration best choice of antibiotic against non-reductive nitrate viable bacteria host reaction as *H. pylori* in percent 66.6% where three isolates of normal felines were more susceptible for ciprofloxacin and amikacin separately (50%) as shown in

table 3 upon inhibition *DNA* gyrase [43] as urease inhibitor of weak urease activity as antitumor activity [44] and binding 30S ribosomes to inhibit protein synthesis [45], respectively reaching to resistance MAR (0.428 & 0.214) as shown in table 4 because felines were specific host attracting to urea and transport by iron and vacuolating cytotoxin associated gene (*vac*A) where its function correlated with type of host and electric charges of organ specificity [46] at colonization level of the species [47]. Resistance of strong urease isolates against ciprofloxacin and amikacin increase with MAR (0.286 & 0.07) that related non reductive nitrate sensitivity (25 & 62.5%) and reductive nitrate sensitivity (25%) from one congested sheep and one milk sample, respectively as shown in table 3 which be suggested according to mis-transcription *RNA* from nitrogen assimilation of any urea either be cytoplasmic urease or extracellular urease [48], evidenced in the present work by negative amplification of v3-v4 region coding for nitrate reducing gram negative bacteria dissimilar to study [49] depending on nitrogen concentration as recorded in study [50]. Sensitivity of ciprofloxacin and amikacin that decline against strong urease biotypes *H. pylori* of normal sheep (33.3%) as shown in table 5 with MAR (0.643) as shown in table 3 may return to host susceptibility to repellant urea by nickel dehydrogenase reaction through rich nickel diet administrated for sheep [51] which be more sensitive than 2 illness felines like constipated and diarrheal cases with MAR (0.857) as shown in table 4 that lock iron elements responsible for regulating transportation adequate amount of urea as main step of *H. pylori* metabolism [52] to cause infection even though positive amplification of *16rRNA H. pylori* resulted from both felines and sheep in equal number of isolates as shown in figure 2.

Conversely to the highest susceptibility of strong urease biotypes (83.3%) related 3 congested sheep, 1 normal and 1 milk and 1 constipated feline against tetracycline and amoxicillin is equal to sensitivity of weak urease isolates related 3 normal felines and 2 illness felines as shown in table 3 & 4 returned to sensitivity non- reductive nitrate 100% across mechanism inhibition of replication or competition with penicillin binding protein for inhibition synthesis of outer membrane protein bacterial cell wall that be homologous and cross react with other denitrifying bacteria [53-54] in gram negative rods or resemble gull winged form as shown in figure 1, which have low sensitivity (25%) as shown in table 3 or resistance against producing nitrate isolates (75%) from excessive utilization to urea diet as natural environmental factor for resistance of almost antibiotics that protect ribosomes from action of tetracycline as biochemically resistance determinants due to energy-dependent efflux of tetracycline or lock urea-based ligand [55-56], so resistance of negative urease and positive nitrate *H. pylori* isolate from normal sheep may occur.

Normal gastric sheep may be resistant reservoir *H. pylori* and transgenic animal for normal felines or human and exaggerate resistance of pan-leukopenia or constipated felines otherwise natural transmission ways of stool of illness felines may occur because its resistance 100% MAR (1.0) as shown in table 5 resulted non-sensitive nitrate producing among nitrate reductive isolates in percent 66.6% with sensitivity (25 -50%) that not produced from weak urease biotypes in percent 50% but sensitivity of non-reductive nitrate biotypes was 12.5% in proportional to sensitivity of strong urease biotypes 16.6% as shown in table 3 across utilization urea to adequate amount of nitrogen assimilated.

H. pylori strong or weak urease biotypes in 50% percent for each of both as shown in table 3 may produce ferritin that showed a wild-type strain grown in an iron-rich environment to aggregate cytoplasm containing iron which not present in mis metal-regulated urea cycle of diarrhea or constipated felines cases or pan-leukopenia to be moderate sensitive (50% & 66.6%) against metronidazole and rifampicin, respectively where mode of their action effect on binding ribosomes as nitro reductase encoding gene [57] and host susceptibility chelation iron receptor [58] for attracting urea, respectively to assimilate nitrogen that resist against nitrate product in percent 75% and sensitive in percent 62.5% & 75%, respectively from non-nitrate reducing biotypes as shown in table 3. The same of ill felines isolates, weak urease isolates from ingestion normal gastric sheep which is more transmissible through the highest resistance antibiotic than weak urease of diarrheal felines with low resistance (0.857) or strong urease from two congested sheep and milk sample with lower resistance (0.5 & 0.071) and (0.286) as shown in tables 4 & 5 upon excess nitro-product or molar urea determination as elements and enzymes that interact with metabolism *H. pylori* [59], making decision of best choice antibiotic especially after measurement of nanomolar urea as weak urease as critical step. To notify exclusion sheep healthy meat that be more susceptible to producing nitrate from urea and resistance to antibiotics, not as congested meat or contaminated milk that be less dangerous in vitro from consuming and recommend that quantification of nitro product of *H. pylori* should be used for programming eradication therapy upon nitrifying or detoxifying *H. pylori*.

Group of Ciprofloxacin, gentamycin, cephazolin and imipenem have sensitivity 25% against -\+ve nitrate reductive biotypes that descend to 12.5 & 0%, respectively against each of penicillin and cephalothin that gathered in other group have descending sensitivity from strong to weak urease biotypes from 33.3 &16.6% to 16.6 & 0%, against each group respectively except ciprofloxacin, were susceptible to two normal feline and sheep plus milk as shown in table 3. Nearly equal to metronidazole, rifampicin & tetracycline and amoxicillin sensitivity 62.5, 75 & 100% respectively against nonreductive nitrate biotypes in equilibrium sensitivity between weak & strong urease biotypes 50%, 66, 6% & 83.3%. Concluded non reductive nitrate biotype is considered marker for sensitivity antibiotics especially when produced from strong urease biotypes that be mode action of almost antibiotics except amikacin followed by ciprofloxacin act on weak urease coded by ribosomes binding, ended by resistance against over binding nitro product in ribosomes of whole biotypes *H. pylori.* Biochemical variations of producing nitrate or utilization

urea plus clinical condition had upper hand on selection the best choice of antibiotic except tetracycline and amoxicillin were susceptible against almost isolates biochemically [55], except one isolate from normal sheep that may be *16srRNA* mutated into resistant strain that need for exclusion from eradication program. Especially after confirmation of presence nitrate reducing bacteria in specimens, using v3-v4 region provided negative result in the present study as shown in table 2 mention role of *H. pylori* independently in sheep and felines.

Then clarithromycin and levofloxacin sensitivity (83.3%) were recommended for illness sheep through 5 strong urease isolates related 3 congested and milk and one gastric normal sheep as shown in table 3 & 5 upon binding 50S ribosome expressed by *23srRNA H. pylori* [60] and inhibition of binding topoisomerase [61], respectively, that was more suitable than sensitivity of 4 weak urease isolates that recovered from 3 normal and one diarrheal feline alone that resist clarithromycin as shown in table 4 where bacteria may be shedded in diarrheal excretion but pan-leukopenia and constipation have strong and weak urease, respectively resist together upon host capability factor of replication DNA over alteration enzymes as gyrase or topoisomerase resulted in change efflux antibiotic [62].

Penicillin and cephalothin have no susceptibility against isolates with MAR (0.714, 0.50 & 0.071) as shown in table 5 except one congested sheep because *H. pylori* is considered as (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species)ESKAPE pathogen to share its antibiotic resistance genes with other bacteria as pseudomonas [41] as commonly recognized with *H. pylori* biofilm that will be discussed in the next research papers.

Sensitivity of antibiotics for whole isolates of felines and sheep in that work was in the descending manner according to principal component analysis of susceptibility antibiotics against *H. pylori* as shown in figure 4: tetracycline, amoxicillin, levofloxacin, clarithromycin, rifampicin, amikacin, metronidazole, ciprofloxacin, gentamycin, cephazolin, imipenem, penicillin, cephalothin and nalidixic.

5. Conclusions

Sensitivity urease *H. pylori* biotypes to antibiotics depends on producing nitrate reductive or non- nitrate reductive through host susceptibility that returned to healthy condition of felines and sheep, and environmental factor of animal product.

Author Contributions

Dr. Mohamed ElSayed Enany, first supervisor support all authors to work interestingly and cooperatively. Dr. Hanaa Mohamed Fadel, second supervisor provided major effort and materials for finishing laboratory methods. Dr. Usama Hassan Abo-Shama, third supervisor performed statistical analysis and prepared the draft manuscript. Dr. Mona Muhammad Mahmoud as corresponding author, performed study design, collected data, interpretated results and wrote paper. Dr. Mohamed Ezzat Abdel gaied Kholief reviewed the abstract, results and references, suggested title, designed tables, add figures and approved the final version of the manuscript in addition to the financial contribution.

Abbreviations

ACE: Animal Care Hospital
AK: Amikacin
AMX: Amoxicillin
AST: Antimicrobial Susceptibility Test
Bp: Base Pair
CN: Cephalothin
CL: Clarithromycin
CZ: Cefazoline
Co2: Carbon Dioxide
CP: Ciprofloxacin
ESKAPE: Enterococcus Faecium, Staphylococcus Aureus, Klebsiella Pneumonia, Acinetobacter Baumannii, Pseudomonas Aeruginosa, and Enterobacter Species
E-test: Epsilometer Test
G: Gentamicin
H. pylori: Helicobacter pylori
IPM: Imipenem
L: Levofloxacin
M: Metronidazole
MAR: Multiple Antibiotic Resistance
MICs: Minimum Inhibitory Concentrations
NA: Nalidixic acid
NCCLs: National Committee for Clinical Laboratory Standards
NO: Nitric Oxide
P: Penicillin G
PBs: Phosphate Buffer Saline
PCR: polymerase Chain Reaction
RF: Rifampicin
SAS: Statistical Software Suite
TBE: Tris\Borate\EDTTA
T: Tetracycline
Thio: Thioglycolate Broth
UV: Ultraviolet
vacA: Vacuolating Cytotoxin Associated Gene
WHO: World Health Organization

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Conflicts of Interest

The authors declare no conflict of interest.

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