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Antibiotic Susceptibility Pattern against Virulence Genes of *Helicobacter. pylori* Isolated from Felines and Sheep

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Abstract | *Helicobacter, H. pylori,* is seasoned common bacterial humans pathogen, possess virulence genes such as vacuolating cytotoxin gene A (*vac*A), cytotoxin-associated gene A (*cag*A) and, (*hrg*A) which have not widely recognized function. The aim of the current study to determine the efficacy of antibiotic therapies of human diseased animal model as felines and sheep infected with virulent *H. pylori* in seasons. A total of twelve *H. pylori* isolates cultivated from samples of stomachs and stool of felines, gastric and milk of sheep. These were previously confirmed by amplification of *16srRNA H. pylori* to be detected by multiplex polymerase reaction against *vac*A, *cag*A, and *hrg*A and bio-typed based on urease and nitrate reduction testes, finding non-nitrate reductive isolates from apparently healthy felines 20% and, nitrate reductive isolates from clinical felines and normal sheep 40% and 20%, respectively as total virulence genes *H. pylori* (*cag**vac**hrg*A) frequency in autumn. Among the highest frequency both of *cag**hrg*A 66.6%, positive nitrate biotypes were highly resistant 85.7 % in autumn through antimicrobial susceptibility test, conversely to non-nitrate isolates are sensitive 35.7% & 57.1% in autumn and winter, respectively especially for felines against levofloxacin, tetracycline and amoxicillin in presence of *vac*A 100%, otherwise deficient *vac*A isolates of sheep; nitrate and non-nitrate biotypes from milk and congested gut in summer and spring, respectively representing *cag*A & *hrg*A 50% have sensitivity 0% till 28.6%. In conclusion, the highest resistance to *cag*A of *H. pylori* in autumn season and the highest sensitivity for *hrg*A in winter season was recorded.

Keywords: *Helicobacter. pylori*, Virulence genes, Antibiotic susceptibility, Felines and sheep

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INTRODUCTION

H. pylori is a pathogenic bacterium, often associated with gastrointestinal diseases rather than benign coexistence, may be associated human and human diseased animal model disease as felines and sheep carrying different virulence genes related variable antimicrobial resistance (Ferrero and Fox, 2001) in variable seasonal incidence (Newell, 2001). Function of virulence genes contribute to the development of *H. pylori* related-diseases (Proença-Modena

et al., 2009) that differ according to environmental factors of climate change (rainy or dry, high or low temperature, infected or hygienic) can influence bacterial prevalence that reflect on multiple antibiotic resistance drug action in vitro as well as vivo (Sailors, 2022) and, morphogenesis that was observed by a scanned electron microscope into rods or coccus (Krzyżek and Gościniak, 2018). While the direct impact of climate change on the expression of specific virulence genes is not well-documented, that could not interpretate seasonality of antimicrobial resistance (Sonnenberg, 2022). For example, the cause of the highest levels of *H. pylori* contamination at 32 ˚C for summer in Iran (7.36%) (Ranjbar *et al*., 2016), followed by (2.1% and 2%) from drinking water samples at 17 ˚C for spring and 16 ˚C for autumn, respectively is the high temperature, disagrees with the cause of coinfection of Egyptian children that increases more in winter than summer which have the highest prevalence of *H. pylori* (4.54%) (Ibrahim *et al*., 2019), depending upon biofilm formation. Thus, cultivation is more standard test *H. pylori* than PCR survey (Ndip *et al*., 2003) that commonly detected by multiplex PCR, in full consideration with biochemical properties of *H. pylori* effect on antimicrobial susceptibility within seasonal variations.

Therefore, the aim of the present work was selecting the best antibiotic for each virulence gene of *H. pylori* in each season of the year.

MATERIALS AND METHODS

Ethical Approval

The animal under experiment were approved from the Research Ethics Committee, Faculty of veterinary medicine, Suez Canal University (Registration number: 2016100) to collect stool swabs from rectum of felines with sterile sticks into sterile tubes with buffer (Sabbagh *et al*., 2019), and collect gastric samples from immediately dead or euthanized felines by intracardiac injection of syringe 3 ml saline (Loeffler, 2018) with knowledge of veterinarian clinicians, and collect gastric and milk samples of sheep immediately after slaughtering, and milking in slaughter houses and farms of Hurghada and Sohag, respectively.

Sampling: During summer (2017) to spring (2021), (6 stomach and 46 stool) were collected after serodiagnosis of 52 felines in Animal care hospital (ACE) of Luxor and BLUE MOON clinician branch of Animal Friendship Social Organization of Hurghada, in addition to 83 sheep samples including (66 gastric and 17 milk) to scrub totally seventy-two gastric samples from apparently healthy slaughtered sheep and felines into saline until arrival for laboratory (Han *et al*., 1995) or thioglycolate broth (Stevenson et al., 2000) and, preserve the second flow of seventeen milk samples into sterile cups in an icebox with refrigerants to transport to the laboratory for centrifugation (Bertino *et al*., 2013).

Sero-examination by stool antigen HPSA for felines and serum latex agglutination IgG *H. pylori* for sheep: As instructed for Kits and labelled by date, number and sign of illness.

Isolation: Under sterile aseptic condition of Bensen flame according to (Bury-mone et al., 2006), enriched samples were inoculated for 36 – 48 hours onto thioglycolate broth (Himedia) India) supplemented with urea under microaerophilic conditions using CampyGen gas kit (10% co_2, 85% N_2) (Oxoid) (CN 0035A), cultured for 5 days at 37c on brain heart infusion BHI agar supplemented with antibiotics (vancomycin (EMC. UK.), and amphotericin B (Astellas Pharma. US).

Biochemical Differentiation

One hundred and thirty-five isolates out of totally collected samples were differentiated biochemically (Harper *et al*., 2003) by oxidase, catalase**,** urease and nitrate reduction, characterized by gram staining into gram negative rods or coccus.

Electron Microscope (E\M) for Scanning Representative Feline Isolates (Golding *et al*., 2016).

DNA extraction of *H. pylori*: According to instruction (QIA amp Kit) from which 1uL was used as the template for DNA amplification after one or two colonies were suspended in an Eppendorf tube from overnight culture on brain heart infusion agar plates, with 20 ml of sterile phosphate buffered saline and, vortexed vigorously for 2 minutes, then boiled in a water bath for 15 minutes, cooled in ice, and centrifuged a 13000 g for 1 minute to transfer the supernatant to another tube.

PCR detection of virulence genes *H. pylori* by using Gene Jet Genomic (Chattopadhyay *et al*., 2004) for DNA purification**,** including 25µL of master mix contained 10ng of the extracted DNA and 10pm of each primer in standard PCR buffer in PCR conditions of virulence genes *vac*A, *cag*A, and *hrg*A primers by thermal cycler (Eppendorf, Hamburg, Germany) as shown in table (A), consisted of an initial denaturation of target DNA at 95˚C for 5 min, followed by 35 cycles of denaturation at 94˚C for 30 s, primer annealing at 52˚C for 1 min, and extension at 72˚C for 1 min (Tiwari *et al*., 2007). The final cycle included extension for 7 min at 72˚C to ensure full extension of the product to be electrophoresed 1.5% (Sambrook *et al*., 1989) and evaluated on a UV transilluminator.

Table (A): Primers used for identification of virulence genes of *H. pylori*

Primer	Sequence	Size	References
brgA	F: TCTCGTGAAAGAGAAT-TTCC	594	(Ando *et al.*, 2002)
	R: TAAGTGTGGGTATAT-CAATC		
cagA	F: GCGATTGT-TATTGTGCTTGTAG	499	(Elrais *et al.*, 2022)
	R: GAAGTGGTTAAAAAA-CAATGCCCC		
vacA	F: ATGGAAATACAACAAA-CACAC	2559	
	R: CTGCTTGAATGCGC-CAAAC		

Antimicrobial susceptibility test (AST): According to the guidelines stipulated by *"NCCLS"*, fourteen discs of antibiotics (Cefazoline (CZ), Gentamicin (G), Tetracycline (T), Clarithromycin (CL), Metronidazole (M), Levofloxacin (Lev), Imipenem (I), Cephalothin (CN), Amoxicillin (AMX), Ciprofloxacin (CP), Amikacin (AK), Penicillin G (P), Nalidixic acid (N), and Rifampicin (RF)) (Basingstoke, Oxoid Hampshire, UK) were tested according to the single diffusion method (Chen *et al.*, 2017) for the determination of the multiple antibiotic resistance (MAR) index by the formula MAR = No. of resistance \Total No. of tested antibiotics (Singh *et al.*, 2010).

Statistical Analysis

Multivariate statistical Package software (MVSP version 3.2), to calculate the genetic similarity and construct the phylogenetic tree for cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic Average) into Nitrate Group A (T & AMX), subgroup 1A (Lev, Cl, R, M, AK, CN, G, CZ), subgroup 2A (I, P, CP & N) as shown in Diagram 2 (B) and, Non-nitrate Group C (nitrate group A + Lev); (T, AMX & Lev) combined sub group C1 (Cl & RF) divided into class 1 & 2 of sub group C1: (M & AK) and (CN), respectively and, subgroup C2 (G, CZ & I) including class 1; (N, P & CP) as shown in Diagram 2 (A) in the following manner of susceptibility as shown in Diagram (1), according to Kovach (2007).

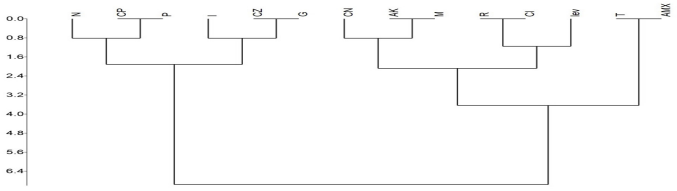

Diagram 1: Phylogenetic tree for efficacy of total susceptibility antibiotics.

RESULTS AND DISCUSSION

Table 1, In winter season, the lowest *H. pylori* isolation 1.23% was recovered from the highest positive survey serological 43.3%. Conversely to autumn, the highest isolation of *H. pylori* 25% was recovered from the highest negative serological survey 55.2%. Total Moderate high percent of isolation 14.2% & 10.5% were recorded from the lowest seronegative survey and lowest seropositive survey 9.7% & 10.1% in summer and spring respectively, compared to 0% isolation from less low negative serological survey of 8 sheep 27.5% in spring. Highest isolation from sheep reported 50% in negative seroprevalence in autumn, followed by 12.5% from positive serological survey in spring then descend into 6.2% from seronegative survey followed by 3.3% from seropositive survey in summer. Highest isolation from felines 50% occurred from seropositive survey in winter followed by 16.6% & 3.1 % from seronegative & seropositive, respectively in autumn that descended to zero isolation in spring and summer. Serodiagnosis is highly and moderate significance in seasons 0.001 & 0.0012 from sheep and felines, respectively as relation of combination diagnostic methods; PCR and serology is moderate significant 0.007 in total seasons but less significant by

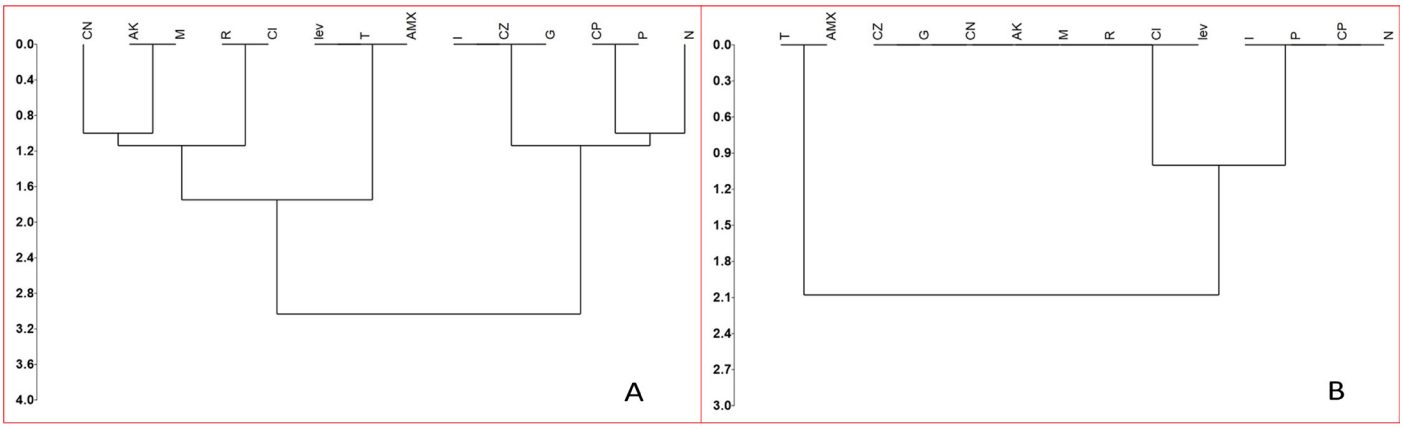

Diagram 2: Phylogenetic tree of efficacy susceptible antibiotics against non-nitrate reductive (A) and nitrate reductive *H. pylori* isolates (B).

Table 1: Seasonal serotypes of *H. pylori* isolate confirmed by PCR in felines and sheep.

Test	+ve	-ve	Total	Serotypes						
				(+)	(-)	(+)	(-)	(+)	(-)	(+)
Serology	(189) S:148 + F:41	(29) S:12 + F:17	(218) S:160 + F:58	Autumn		Summer		Spring		Winter
				S:11 F:32	S:4 F:12	S: 30 F: Nil	S: 16 F: 5	S: 16 F: 3	S :8 F: Nil	S:75 F:6
P-value serology % of sheep	< 0.001 (***)			8.3	14.3	22.7	57.1	12.1	28.6	56.8
P-value serology % of felines	0.0012 (**)			78	70.6	0.0	29.4	7.3	0.0	14.6
Total (%)	86.6	13.3	S:73.3 F:26.6	23.5	55.2	13.8	9.7	10.1	27.5	43.3
PCR	7 S:3 + F:4	5 S: 3 + F: 2	12 S:6 + F:6	S:0 F:1	S: 2 F: 2	S: 1	F:0 S: 1	F:0 S: 2	S:0	S: 0 F:3
P-value PCR % of sheep	0.035 (*)			0.0	66.7	33.3	33.3	66.7	0.0	0.0
P-value PCR % of felines	0.028 (*)			25	100	0.0	0.0	0.0	0.0	75
Total (%)	3.7% S: 2.0 F:9.7	17.2% S: 25 F: 11.7	5.5% S: 3.7 F: 10.3	2.3% F:3.1%	25% F: 16.6 S:50	S: 3.3	14.2% S: 6.2	10.5% S:12.5	0	1.23% F:50
Total ratio No. (%)		189:7 (96.4:3.6)	29:5 85.3:14.7	P-value: 0.007 (**)						

***: Highly significance. **: Moderate significance. *: Less significance. S: Sheep. F: Felines.

Table 2: MAR of resistant virulence genes recovered from sero-examined *H. pylori* infected animals.

Total virulence genes of biotypes H. pylori isolates in seasons	Season						
	Autumn (5\12) 41.6%		Winter (3\12) 25%		Spring (2\12) 16.6%	Summer (2\12) 16.6%	
	virulent				Avirulent		
	Nitrate	Non nitrate	Non nitrate avirulent	Non Nitrate virulent	Non nitrate	Nitrate	Non nitrate
MAR of *H. pylori* isolate recovered of clinical cases	Clinical cases						
	Constipated Panleukopenia felines 0.857 Normal sheep (1.0)	Normal sheep 0.643 & prolapsed uterus feline 0.214	dead\ Diarrhea felines 0.428\ 0.857	Normal feline 0.428	Normal Congestion sheep 0.714\0.5	Milk 0.286	Congestion sheep 0.071
*cag*A	3	2	1	1	1	1	0
*vac*A	3	2	2	1	0	0	0
*hrg*A	3	2	1	1	2	1	1
No. PCR of serology +ve	0	1\43	3		2\19	1\30	0
Total ratio %	0	2.3:97.7	None results of negative serology		9.5:90.5	3.2:96.8	0
No. PCR of serology -ve	3	1	Nil		0\8	0	1\16
Total ratio %	4\16 (20:80%)		No ratio		0:100	0	5.9:94.1
Sign (P-value)	0.014				NS (0.365)	NS (0.659)	

PCR 0.035 & 0.028 from sheep and felines, respectively especially in autumn seasons as shown in Table 2**,** with less significance 0.014 and no significance of combination diagnostic methods in other seasons 0.365 & 0.659 that was differentiated by culture and bio typing.

Table 2 and 3, No significance of seasons 0.632 & 0.723 on frequency of virulence genes, as shown in Table 2, but bio typing *H. pylori* has highly significance 0.001 on antibiotic susceptibility for virulence genes in seasons, as shown in Table 3. *Cag\vac\hrg*A nitrate reductive *H. pylori* isolates, in percent 20% have no susceptibility against normal sheep with MAR (1.0) among autumn isolates 41.6% as shown in Table 2, compared to *cag\vac\hrg*A *H. pylori* in percent

40% from clinical felines constipated and pan-leukopenia in autumn have the highest susceptibility against nitrate group A (tetracyclines and amoxicillin), as the same to susceptibility nitrate biotype deficient *vac*A *H. pylori* from milk of sheep in summer with MAR (0.857 & 0.286), respectively in addition to, subgroup 1A plus imipenem in highest and moderate susceptibility 100 & 50% in summer against nitrate and non nitrate reductive isolates from milk and gut sheep, respectively than lesser susceptibility deficient *hrg*A *H. pylori* among percent 25% isolates in winter against non-nitrate reductive antibiotic group C1 (rifampicin and clarithromycin) plus their subclasses (metronidazole and amikacin) and cephalothin for suddenly dead felines, have MAR 0.428, respectively in nearly equal to the lowest susceptibility highly virulent isolate of normal sheep in frequency 20% have MAR 0.643 in autumn, as shown in Table 3. Lowest susceptibility of *cag**vac**hrg*A nitrate reductive *H. pylori* in autumn against nitrate antibiotic group (T & AMX, Lev, Cl, R, M, AK, CN, G, CZ, I, P & CP) have MAR 0.857 than susceptibility of *vac*A non-nitrate reductive biotype of diarheal, suddenly dead and normal feline isolates with MAR (0.857 & 0.428) against ciprofloxacin especially as class 1 of sub group C2, followed by group C and subgroup C1 and classes 1 & 2 in winter. The susceptibility of moderate incidence *cag*A 50% in spring increase against non-nitrate antibiotic group group C and clarithromycin as one of subgroup C1 with MAR 0.714, both of isolates from summer and spring in percent 16.6%, were susceptible against group C, followed by calarithromycin then rifampicin as subgroup C1 then class (1) of subgroup C.The highest susceptible *H. pylori* present in summer carry *hrg*A non nitrate reductive bioype isolate have MAR 0.071 than susceptibility *hrg*A that present in spring have MAR 0.5 from congested gut of apparently healthy sheep.

Table 4, Highest susceptibility of whole virulence genes against non- nitrate antibiotic group (C); tetracycline, amoxicillin and levofloxacin is 100% in winter & spring as well as clarithromycin as one of subgroup C1 represent 66.6% in spring in addition to class 1 of subgroup C1; metronidazole and amikacin and, subgroup C1 plus suscepti

Table 3: Number sensitive antimicrobial disc against percent seasonal occurrence virulent biotype *H. pylori* isolate from felines and sheep.

	Nitrate			**Non nitrate**			
	Autumn		**Summer**	**Autumn**	**Spring**	**Summer**	**Winter**
*cag*A	(2) 40%	(1) 20%	1 (100%)	(2)40%	50%	0	(2) 66.6%
*vac*A	(2) 40%	(1) 20%	0	(2)40%	0%	0	(3) 100%
*hrg*A	(2) 40%	(1) 20%	(1) 50%	(2)40%	(2)100%	(1) 50%	(2) 66.6%
P-value	1.00	1.00	0.606	1.00	0.173	0.659	0.525
Sig.	**0.632 (NS)**			**0.723 (NS)**			
	F:2	S:1	S:1	F:1 & S:1	S:2	S:1	F:3
N	0	0	0	0	0	0	0
CP	0	0	0	0	0	1	0
P	0	0	0	0	0	1	0
I	0	0	0	1	0	1	0
CZ	0	0	1	1	0	1	0
G	0	0	1	1	0	1	0
CN	0	0	1	1	0	1	2
AK	0	0	1	1	1	1	2
M	0	0	1	1	1	1	2
R	0	0	1	2	1	1	2
Cl	0	0	1	2	1	1	2
lev	0	0	1	2	2	1	3
T	2	0	1	2	2	1	3
AMX	2	0	1	2	2	1	3
Sensitive	2		0	5	4	0	8
Resistant	0		10	6	3	13	0
Highly Resistant	12		4	3	7	1	6
MAR	0.857	1.0	0.286	0.643, 0.428 & 0.214	0.714 & 0.5	0.071	0.428 & 0.857

Table 4: Percent seasonal antimicrobial susceptibility biotypes *H. pylori* isolate associated virulence genes.

AB discs	Nitrate					Non nitrate								
	Autumn			Summer		Autumn			Spring		Summer	Winter		
	C:3	V:3	H:3	H:1	C :1	C:2	V:2	H:2	C:1	H: 2	H:1	C:2	H:2	V:3
T	60	60	60	50	100	40	40	40	100	100	50	100	100	100
AMX	60	60	60	50	100	40	40	40	100	100	50	100	100	100
Lev	0	0	0	50	100	40	40	40	100	100	50	100	100	100
CL	0	0	0	50	100	40	40	40	100	100	50	50	50	66.6
R	0	0	0	50	100	40	40	40	0	50	50	50	50	66.6
M	0	0	0	50	100	20	20	20	0	50	50	50	50	66.6
AK	0	0	0	50	100	20	20	20	0	50	50	50	50	66.6
CN	0	0	0	50	100	20	20	20	0	0	50	50	50	66.6
G	0	0	0	50	100	20	20	20	0	0	50	0	0	0
CZ	0	0	0	50	100	20	20	20	0	0	50	0	0	0
I	0	0	0	50	100	20	20	20	0	0	50	0	0	0
CP	0	0	0	0	0	0	0	0	0	0	50	0	0	0
P	0	0	0	0	0	0	0	0	0	0	50	0	0	0
Sensitive	14.3			0		35.7			28.6		0	57.1		
Resistant	0			71.4		42.9			21.4		92.9	0		
Highly Resistant	85.7%			28.6		21.4			50		7.1	42.9		
P-value	< 0.001 (***)					< 0.001 (***)								
MAR	1.0 & 0.857			0.286		0.214 & 0.643			0.714 & 0.5		0.071	0.428 & 0.857		

bility class 2 in winter while moderate susceptibility *hrg*A represent in spring 50% against one of subgroup C1 as rifampicin and class 1 but *hrg*A in summer have also moderate susceptibility 50% against subgroup 2A, especially against nitrate reductive *H. pylori*. The lowest susceptibility of the highest virulence gene of non-nitrate reductive isolates was 40% in autumn against group C; tetracycline, amoxicillin, and levofloxacin and subgroup C1; clarithromycin, and rifampicin. Nitrate and non-nitrate virulent *H. pylori* has highly significance < 0.001 on susceptibility antibiotics in seasons.

Table 5, Winter is season of susceptibility levofloxacin, clarithromycin and rifampicin in 75-66.7% from virulent *H. pylori* of felines than susceptibility deficient *vac*A and moderate *cag*A from sheep (40-50%) in summer, in addition to amikacin & metronidazole 66.7%, cefazolin & gentamycin and cephalothin plus imipenem 100% but highly virulent isolates in autumn have total sensitivity; 50%, 41.7%, 25% and 16.7%, respectively. Deficient *vac*A isolates from sheep in summer have the low moderate susceptibility against tetracycline and amoxicillin 40% than felines 50%, especially levofloxacin and clarithromycin 25-33.3%, respectively from highly virulent isolates in autumn which increase to 50% in summer against rifampicin for deficient *vac*A and moderate *cag*A sheep isolates in total susceptibility metronidazole and amikacin 50%, reporting the highest susceptibility especially, ciprofloxacin and penicillin for deficient *vac*A isolates of sheep is totally 8.3% increase than *vac*A isolates of felines in summer but in the same susceptibility cephazolin & gentamycin and imipenem in total 16.7 and 25%, respectively in all seasons.

Helicobacter is a seasoned pathogen (Ahmed *et al*., 2009) like *Helicobacter pylori* (bcc3) among oxygen-consuming bacteria express only one oxygen reductase (André *et al*., 2021) in extracellular bacteria colonization on host tissues or within biofilms by aero protection strategy (André *et al*., 2021), listed as 'priority pathogens' by the WHO (Suzuki *et al*., 2022). Under increased temperature, pathogenic bacteria producing nitrogen oxide related by *cag*A as powerful virulence properties for hydrogen metabolism of *H. pylori* from nitrate reduction for antimicrobial resistance (Wang *et al*., 2016). Less significance of diagnosis *H. pylori* by PCR from highly and moderate significance of seroprevalence 0.001 & 0.0012 was 0.035 & 0.028, respectively as shown in Table 1, that be commonly investigated in autumn season from clinical cases felines and normal sheep have MAR 0.857 and 1.0 in percent of isolates 41.6% as shown in Table 2, that detected by highly significance diagnostic methods combination from PCR and serodiagnosis in 0.014 as shown in Table 3, which agree with highly significance 0.0001 of low susceptibility 60% and the highest antibiotic resistance of nitrate *H. pylori* biotypes among incidence *cag**vac**hrg*A 40% & 20% in non-significance 0.0632 against nitrate group A antibiotics as shown in Table 4

Table 5: Total Antimicrobial susceptibility of seasonal *H. pylori* isolates from felines and sheep

	Susceptible Felines in				Susceptible Sheep						Total sensitivity		Total resistance	
	A		W		A		S		SP					
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
N	0	0.0	0	0.0	0	0	0	0	0	0	0	0	12	100
CP, P	0	0.0	0	0.0	0	0	1	100	0	0	1	8.3	11	91.7
I	1	100	0	0.0	0	0	1	100	0	0	2	16.7	10	83.3
CZ, G	1	100	0	0.0	0	0	2	100	0	0	3	25	9	75.0
CN	1	50.0	1	50.0	0	0	2	100	0	0	4	41.7	7	58.3
AK, M	2	66.7	1	33.3	0	0	2	66.7	1	33.3	6	50	6	50.0
R	1	33.3	2	66.7	1	25	2	50	1	25	7	58	5	41.7
CL	1	33.3	2	66.7	2	40	2	40	1	20	8	66.6	4	33.3
Lev	1	25.0	3	75.0	2	40	2	40	1	20	9	75	3	25.0
T & AMX	3	50.0	3	50.0	2	40	2	40	1	20	11	91.7	1	8.3
P-value	0.757				0.948						0.0038			
Sig	NS				NS						**			

A: Autumn. S: Summer. SP: Spring. W: Winter.

that bind nitrate metabolites perhaps resulted from immune physiological mechanisms. Thus depending on nitrate reduction of isolates upon *cag*A status of *H. pylori*, the highly significance and less significance of each serology and PCR prevalence 0.0001 & 0.35 as shown in Table 1**,** provided the highest susceptibility nitrate and non-nitrate antibiotic group 100% in non-significance 0.0632 & 0.0723, respectively for different incidence of *cag*A 66.6%, 50% and (100% and zero) in winter, spring and summer, respectively, especially from sheep isolates which were tested in non-significance 0.659 & 0.365 of diagnostic methods combination; PCR and serodiagnosis in incidence 16.6% in spring and summer as shown in Tables 2 and 3, may be differentiated by energy expenditure from acquisition hydrogen as molecular source present from other bacteria (Benoit *et al*., 2020) as from higher contamination in summer making susceptibility nitrate and non-nitrate antibiotic group as penicillin group in less MAR 0.286 for nitrate biotype *cag*A from milk of apparently healthy sheep where MAR descend more for deficient *cagA**vac*A non-nitrate biotype *H. pylori* from congested gastric sheep to be susceptible 50% in presence of 50% *hrg*A have MAR 0.071, as shown in Table 3. While higher resistance non-nitrate *H. pylori* perhaps present because lockage moisture in spring releasing high energy reductants (MacKichan., 2004) for avoiding oxygen diffusing within host organs by oxygen reductases from the bacterial genome, which is probably insufficient to assess their function and role during infectious processes (André *et al*., 2021), resulted MAR (0.714 and 0.5) for *hrg*A 50% and 100%, respectively and *cag*A 50%, theoretical moderate and full susceptibility as shown in Table 4. The seasonality of *H. pylori* depending energy of *H. pylori* metabolites that does not possess genes for a nitrate reductase (Marais *et al*., 1999) correlate with immunity host causing carcinogenesis in an animal model (Lu *et al*., 2018), interpretating a decreasing susceptibility between spring and autumn in 0%, 20% 40% and 50%, compared moderate susceptibility in spring for non- nitrate reductive deficient *cag*A *H. pylori* from apparently healthy sheep against metronidazole, amikacin, and rifampicin 50% with exception full susceptibility clarithromycin, levofloxacin, tetracycline, and amoxicillin 100% as shown in Table 4, and resistance gut infected clinical felines as panleukopenia and constipated felines with 60% susceptibility of nitrate biotype against tetracycline and amoxicillin in presence *vac*A prevalence 40% in autumn in addition to full resistance 100% of isolate from normal sheep in MAR 1.0 in prevalence *vac*A 20% that the same prevalence for non-nitrate biotype from normal sheep and uterine prolapsed feline in MAR 0.643 & 0.214, respectively in nearly moderate low susceptibility 20-40% as shown in Table 3. Auto urea transporter gene *vac*A incidence releasing energy utilizing nitrogen where urease gene metabolism functions based upon high protein diet in that season (Obitsu *et al*., 2011) when autumn is a wet season under high ambient temperature may provide favorable conditions for *H. pylori* proliferation in animals' gastrointestinal tracts which is reflected by susceptibility of higher contamination observed in September and October in Dhamar (Almashhadany *et al*., 2023).

The lower *vac*A incidence 1.23% in total felines 50% from positive serological survey 43.3% in winter as shown in Table 1**,** related the highest susceptibility 100% of non-nitrate antibiotic group C; tetracycline, amoxicillin and levofloxacin for the highest incidence of *hrg*A 100% than the moderate susceptibility of metronidazole, amikacin, ciprofloxacin,

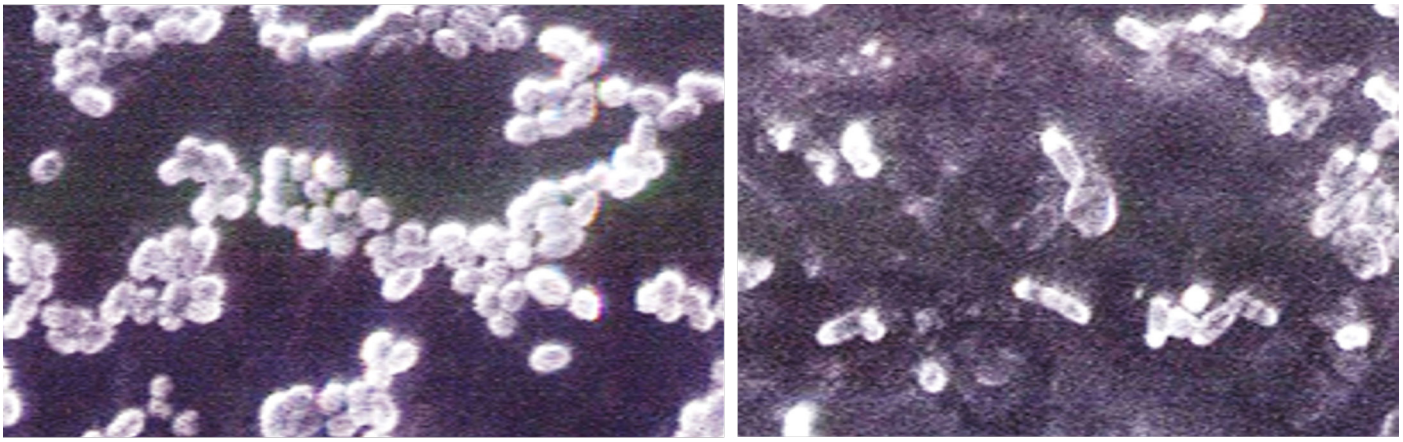

Figure 1: Elecron microscope of felines isolates.

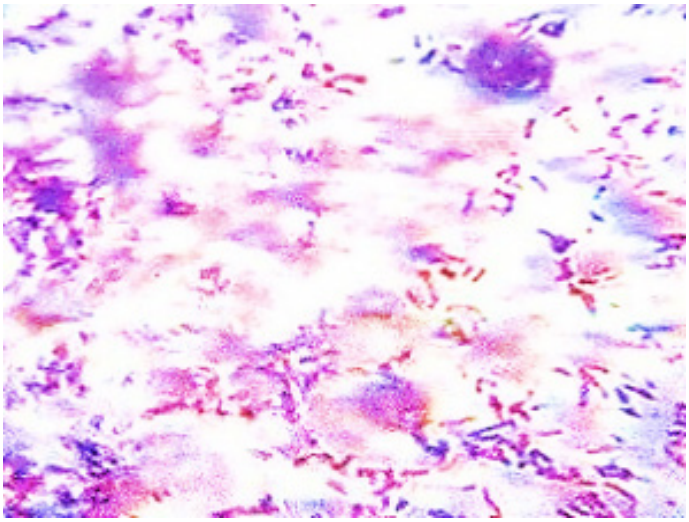

Figure 2: Gull winged gram-stained *H. pylori* isolate.

rifampicin and clarithromycin in 50 & 66.6% for incidence *cag**hrg*A 66.6%, as shown in Table 3 when seasonal patterns as endemic health factors reflect on colonizing the inflamed gut of diarrheal felines in winter through frequency *vac*A 100% with no association *cag*A or nitrate reduction in high resistance MAR 0.857 caused limiting antibiotic absorption by reduced metabolic activity and increasing its chance of survival led to decreasing the amounts of cytoplasm and proteins, which gives a higher resistance (Rosli *et al*., 2024), especially gentamycin and group penicillin, similar to the Venezuela study where *H. pylori* infection frequency was significantly higher during the rainy season (96%) than during the dry season (Domínguez-Bello *et al*., 2002), or whenever were caused by low bacterial load and low expression of Epidermal Growth Factor (EGF) & Heat Shock Protein (HSP) induced by ribosomes from normal or dead felines related lockage immunity physiological process (Yuan *et al*., 2015), respectively in moderate lower resistance 0.428 as shown in Table 3. At colonization level of urea transporters *vac*A in gut tissue species as felines (Joosten *et al*., 2016) or membrane-associated nickel-containing hydrogenases (Wang *et al*., 2016) as hydrogen-utilizing normal and congested gut sheep, energy generation for binding and then "splitting" of hydrogen gas upon electric charges of organ specificity (Domnin *et al*., 2022) continued energy metabolism by oxygen reductase (Sah *et al*., 2023) like *H. pylori* coccoid of constipated felines present in autumn in higher MAR 0.857 as representative isolates by electron microscope E\M otherwise rods of normal felines with MAR 0.428 in winter as shown Figure (1 and 2) and Table (2) similar to *H. pylori* coccoid that increased pyruvate oxidoreductase POR expression in the fall of 2003 (Zeng *et al*., 2008). Compared between total *cag**hrg*A 10.5% from 10.1% seropositive animals of 12.5% sheep in spring, and low occurrence *H. pylori* positive and negative PCR in sheep 3.3% and 14.2% from seroprevalence 13.8% and 9.7%, respectively forming total PCR positive and negative test 3.3% & 6.2% in absence of *vac*A in summer, as shown in Table (1), moderate and full susceptibility *H. pylori* was recorded in 50-100% as shown in Table (3), similar to more resistance of *hrg*A*cag*A *H. pylori* nitrate reductive biotypes from milk in summer is being 0.286 otherwise the lowest resistance of deficient *cag*A non-nitrate reductive biotypes from congested sheep 0.071 as shown in Table (2 and 3), may be because elevated metabolites which could be part of antibiotic resistance mechanisms more expressed by high metabolic activity (Lin *et al*., 2023) that are essential to sustain energy-demanding AMR processes; such as cell-wall changes and efflux pump overexpression (Kok *et al*., 2022). However, susceptibility followed by 91.7%-16.7 percentage in autumn against all antibiotics except penicillin and cephalothin as shown in Table (5) while moderate low resistance 42.9% and 21.4% susceptibility from 23.5% seropositive felines and sheep prevalence monitored in autumn as shown in Table (4), against non-nitrate reductive *H. pylori vac*A from normal sheep and total felines as uterine prolapsed feline 3.1% as shown in Table (1) according to the ability to conserve energy in form of adenosine triphosphate (ATP) through generation of a transmembrane proton motive force (Prakasham and Kumar., 2019) as a compact and high-en-

ergy substrate for respiratory (Wang *et al*., 2016), followed by oxidation of ammonia resulted in denitrifying and antibiotic susceptibility or may be due to releasing a hydrogen molecule as a source of energy (Olson and Maier, 2002) leading to nitrate reduction resulted in binding ribosomes and antibiotic resistance. Concluding autumn season have *vac*A *H. pylori* incidence 41.6% as shown in Table (2) is season for antibiotic resistance in the present study.

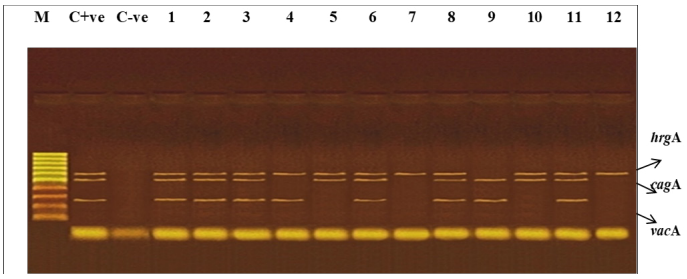

Figure 3: Agarose gel electrophoresis of multiplex PCR *vac*A (259), *cag*A (499) and *hrg*A (594) virulence genes of *Helicobacter pylori* strains. Lane M: 100bp ladder as molecular DNA marker. Lane C +ve: Control positive. Lane C-ve: Control negative. Lane from 1 to 8 and from 10 to 12: Positive *H. pylori* strains for *hrg*A at 549 bp. Lane 1, 2, 3, 5, 6, 8, 9, 10 and 11: Positive *H. pylori* strains for *cag*A at 499bp. Lane 1, 2, 3, 4, 6, 8, 9 and 11: Positive *H. pylori* strains for *vac*A at 259bp.

Susceptibility of metronidazole, amikacin, a group of penicillin, gentamycin, and imipenem against each virulence gene of non-nitrate biotype *H. pylori* from apparently healthy felines and normal sheep of autumn in percent 20%, as shown in Tables 2 and 3, has MAR (0.214 and 0.643), especially against non-nitrate antibiotic group C and subgroup C1, as shown in Table (4) even though other one normal sheep is resistant 100%, that interpretated perhaps through the extremely cold temperature that increases the gastric acid secretion (Liu *et al*., 2006) when the ratio of urea-N production to digestible N was increased after decreasing gut urea-N entry in presence *vac*A (Obitsu *et al*., 2011) to represents the higher incidence of *hrg*A 66.6% with the highest susceptibility in range 91.7%-41.7% in highly significance 0.0001 against prevalence felines in winter 25% as shown in Table (2 and 5) than *hrg*A incidence 40% in autumn was represented through the extremely hot temperature as Nitric Oxide Synthase in the gastric mucosa by serine protease High temperature requirement *HTr*A protein (Zarzecka *et al*., 2019) in total nitrate *vac*A gene incidences 60% in autumn of felines and sheep prevalence by PCR 25% recovered from seronegative 55.2% in total genetic felines infected with *H. pylori* incidence 2.3%, providing full high resistance in moderate significance 0.0012 but in total less significance 0.0028 as shown in Table (1), concluding *hrg*A is the highest susceptible virulence gene to antibiotics in presence *vac*A, especially in winter as reported in the present study.

All seasons have the highest resistance that decrease gradually with exception summer when highly susceptibility against sheep ranging between 100%-16.6 percentage with exception imipenem and group of penicillin, susceptibility in followed by spring and autumn 100%-50%. Then resistance is ascending in spring against metronidazole, amikacin, ciprofloxacin, rifampicin, clarithromycin and levofloxacin 16.6% as shown in Table (5), that specifically do not reveal any resistance against tetracycline and amoxicillin in autumn and winter as shown in Diagram (1) in total moderate significance 0.0038 of sensitivity and resistance but with non-significance for felines and sheep 0.757 & 0.948, respectively as shown in Table (1). So, the present survey of our seasonality results driving no need of antibiotic eradication program on *H. pylori* in summer in contrast to autumn and spring, which is being against biochemical function in presence of virulence genes than its genetic resistance in highly significance of combination PCR and serology 0.0007 as shown in Table (2), in proportional to first study investigating gene expression changes (Upadhyay *et al*., 2023). Sparacino-Watkins *et al*., (2014) confirmed the importance of total seasonality toward the global respiration nitrogen cycle that metabolize nitrogen as mode action of resistance microbes for energy transduction, detoxification, or assimilation.

CONCLUSIONS AND RECOMMENDATIONS

Seasonal follow up of antibiotic therapy programming against nitrate reaction is considered diagnostic survey of each virulence gene of *H. pylori* till quantifying energy expenditure from oxygen reduction level (precursor of metabolites) within infected cells as the next challenge.

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Dr. Mohamed El Sayed Enany, first supervisor supports my conceptualization with valuable comments to work interestingly and cooperatively for conceptualization. Dr. Hanaa Mohamed Fadel, second supervisor provided materials for finishing laboratory methods. Dr. Usama Hassan Abo-Shama, third supervisor performed reviewed the abstract, results and references, Dr. Mona Muhammad Mahmoud as corresponding author, study design, collected data, interpreted results in statistical analysis and wrote

paper. Dr. Mohamed Ezzat Abdel gaied Kholief revised the draft manuscript, suggested title, designed tables, add figures and approved the final version of the manuscript in addition to the financial contribution.

NOVELTY STATEMENT

Grouping antibiotic susceptibility for each virulence gene of (nitrate reductive) bio-types H. pylori is newly reported against highly resistance cagA in autumn and highly susceptibility hrgA in winter that be interpretative in different view through the present study.

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IRB Approval
The study was approved from the Research Ethics Committee, Faculty of veterinary medicine, Suez Canal University (Registration number: 2016100). As recorded under Ethical Approval.

Conflict of Interest
There is no conflict of interest declared by the authors.

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