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APPLICATION OF CLASSICAL-PCR, NESTED-PCR AND SEMINESTED-PCR IN IDENTIFICATION OF HELICOBACTER SPECIES COLONIZING GASTRIC MUCOSA IN DOGS WITH GASTRITIS

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Introduction. Colonization of dogs' gastric mucosa with *Helicobacter* spp. is common. Based on the conducted research it was demonstrated that microorganisms of *Helicobacter* morphology colonizing the gastric mucosa of dogs (GHLO – gastric *Helicobacter*-like organisms) included: *Helicobacter felis*, *Helicobacter heilmannii*, *Helicobacter bizzozeronii*, *Helicobacter bilis*, *Flexispira rappinii* and *Helicobacter salomonis* [23, 25, 28, 30, 34]. These bacteria were found in the stomach of healthy animals and the stomach affected by the disease process. They were observed in the cardia, the fundus, the corpus as well as the pylorus of this organ [10, 11, 13, 14, 23, 25, 26, 27, 35].

In the diagnosis of the gastric mucosa infections with *Helicobacter* spp. in humans and animals non-invasive and invasive methods are applied. The non-invasive methods include: a breath urease test and serological examinations. The invasive methods are based on the laboratory examination of the gastric mucosa bioptates and they include: a rapid urease test, direct bacteriological preparation, microbiological culture examinations, histopathological examinations (a light and electron microscope) and examinations using polymerase chain reaction (PCR) [4, 8, 17, 12, 16, 18, 21, 22, 31, 32, 36].

Task, the aim of the article The aim of the study was to apply a classical-PCR, nested-PCR and seminested-PCR in detection of gastric mucosa infections in dogs with *gastritis* caused by *Helicobacter* spp. microorganisms and determination of their species.

Material and methods The research included 137 dogs, of different breed and sex, aged from 2 months to 17 years, with dyspeptic signs indicating the stomach diseases (vomiting of different character, decreased appetite, anorexia, variable appetite, progressing body weight loss, *fetor ex ore*, painfulness in the stomach area). This group also entailed the animals not manifesting the above clinical signs, in which during gastroscopy macroscopic changes in the mucosa indicating gastritis were observed. The gastric mucosa bioptates were collected for PCR examination from all the animals.

For detection and species determination of *Helicobacter* strains: classical-PCR, nested-PCR and seminested-PCR were used. For DNA isolation the technique of reversible bond on silicone deposits was applied.

DNA isolation from the collected material

For DNA isolation of *Helicobacter* spp. from examined gastric mucosa bioptates columns and a „QIAamp DNA mini" (QIAGEN) set of reagents were used. These materials were replaced by „Genomic mini" (A&A BIOTECHNOLOGY) sets after having proved exchangeability of both

tests and a simultaneous slight increase in DNA isolation effectiveness. The procedure of DNA isolation followed the recommendations of both producers.

Starters. In designing specific starters for *ureB* genes information about regions of their location, given by Neiger et al. [19] and nucleotide sequences from GenBank data base: L25079 for *Helicobacter heilmannii*, M60398 for *Helicobacter pylori* and X69080 for *Helicobacter felis* were used.

The location of starters was modified based on own computer analyses findings and results of preliminary PCR reactions. Additional starter pairs, allowing conduction of nested-PCR reactions, were designed to improve specificity and sensitivity. The detailed data of location of these starters on sequences from the data base are presented in Tab. 1.

Tab. 1.

Detailed localization of specific startes for *ureB* genes [*Neiger et al. 1998].

species	starter	localization of starter on original sequence	original sequence
Helicobacter heilmannii	Heil2F	984-1003	L25079
	Heil2R	1557-1536	
	W2heilF	1097-1115	
	W2heilR	1481-1459	
Helicobacter pylori	PylNF	3379-3400	M60398
	PylNR	5089-5068	
	WpylF	3931-3952	
	WpylR	4620-4598	
Helicobacter felis	FelF*	43-67	X69080
	FelR*	1190-1168	
	WfelF	154-173	
	WfelR	1045-1024	

Nucleotide sequences of all the starters designed in the present study and selected from literature are presented in Tab. 2.

Wykrywana sekwencja Detected sequennce	Wykrywany gatunek Detected species	Typ reakcji PCR Type of PCR reaction	Etap reakcji PCR Stage of PCR reaction	Starter Starter	*Sekwencja nukleotydowa startera* w orientacji 5'-3' Nucleotide sequence of starter in orientation 5'-3'	Produkt PCR (pz) Product PCR
gen ureB gene ureB	*H. heilmannii*	nested	pierwszy	Heil2F Heil2R	GATAAAGTGCGCTTGGGCGA GGTCAATGAGAGCAGGTTCAAA	574
			drugi	W2heilF W2heilR	ACAAACCAACAGCCCCAGC GCGGAGCATTTCTTTTAAGTTCC	385
	H. pylori	nested	pierwszy	PylNF PylNR	ATGAAAAAGATTAGCAGAAAAG CCTAGAAAATGCTAAAGAGTTG	1711
			drugi	WpylF WpylR	GCGGCTGAAGAATATTCTATGA CGCTGGGTTAATGGTGTATTTAG	690
	H. felis	nested	pierwszy	FelF FelR	ATGAAACTAACGCCTAAAGAACTAG GGAGAGATAAAGTGAATATGCGT	1148
			drugi	WfelF WfelR	CTCATTAGCGGGCGTGTGAT CAATCTTGCCGTCTTTAATCCC	892
16S rDNA	*Helicobacter spp.*	seminested	pierwszy	HelF HelR1	CGTGGAGGATGAAGGTTTTA TACACCAAGAATTCCACCTA	284
			drugi	HelF HelR2	CGTGGAGGATGAAGGTTTTA AATTCCACCTACCTCTCCC	275
	H. bizzozeronii *H. felis* *H. salomonis*	klasyczna	jeden	CAR577F CAR636R	TGCGTAGGCGGGGTTGTAAG CAGAGTTGTAGTTTCAAATGC	78
	H. bizzozeronii	klasyczna	jeden	Bi1F Bi2R	AACCAAYAGCCCCAGCAGCC TGGTTTTAAGGTTCCAGCGC	373
	H. salomonis	klasyczna	jeden	T3B HT135R	AGGTCGCGGGTTCGAATCC ACCAACTGGGCTAAGCGACC	134

Tab. 2. Tabulation of starters and PCR reaction programmes

The starters synthesis was carried out by PAN in Warszawa.

Positive controls

As positive controls of nested-PCR reactions, DNA isolated from *Helicobacter pylori* J99 [1] and *Helicobacter heilmanni* strains, obtained at Medical University in Wroclaw, was used.. *Helicobacter felis* DNA was isolated in Institute of Genetics and Microbiology University of Wrocław.

Electrophoretic division of PCR product

PCR reaction products were divided electrophoretically in 0.9% agarose gel in TBE buffer in the presence of etidine bromide (0,25 μg/ml). Electrophoresis was carried out under voltage 140 V. pUC-MIX8 (Fermentas, SM0301) and DNA λ phage digested by *Eco*RI and *Hind*III enzymes (Fermentas, SM0191)were used as a mass marker. Gels were photographed in UV light 300 – 320 nm in length.

Thermocycler work programmes

All the PCR reactions were carried out in DNA-engine PT200 (JM Research, USA) thermocycler. The following work programmes were applied:

Species specificity of ureB genes fragments amplifying starters

At the design stage the starters' species specificity for detection of *ureB* genes was checked by computer comparison of their sequences with sequences in GenBank data base, using BLASTIN 2.2.13 programme [2]. In order to check experimentally the starters' species specificity the nested-PCR reactions using Heil2F, Heil2R, W2heilF, W2heilR starters and DNA isolated from *Helicobacter heilmannii*, *Heliconacter pylori* and *Helicobacter felis* were performed. Similar reactions were conducted with the remaing starters sets for *ureB* genes.

Results. The research into checking starters' species specificity showed that all the starters met the specificity criterion. PCR products were obtained only in the reactions in which appropriate, specific starters were added to a given DNA matrix (Tab. 2.). The results of these reactions are presented in Fig. 1, 2 and 3.

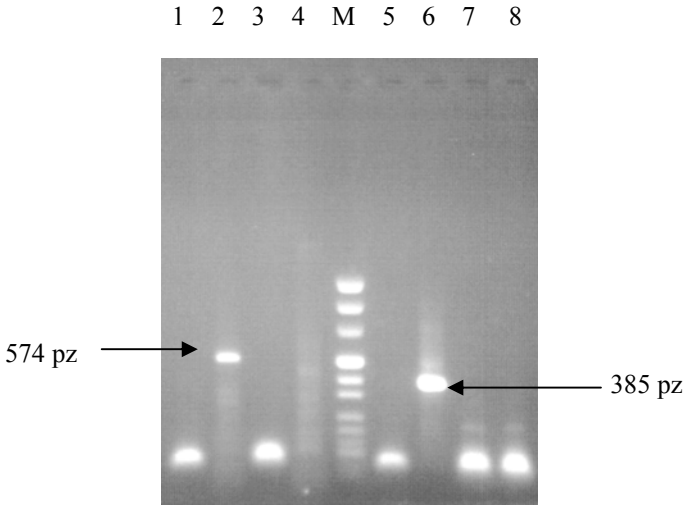


Fig. 1. Species specificity of starters amplifying *ureB Helicobacter heilmannii* gene fragments. Pathways 1 and 5 – negative reagent controls without DNA. First stage of nested-PCR reaction with Heil2F and Heil2R starters and *Helicobacter heilmannii* DNA (pathway 2). Second stage of reaction with W2heilF and W2heilR starters and *Helicobacter heilmannii* DNA (pathway 6); *helicobacter felis* (7) and *Helicobacter pylori* (8). M – mass marker, pUCMix8 (Fermentas, SM0301). Product characteristics for subsequent nested-PCR stages are marked with arrows.

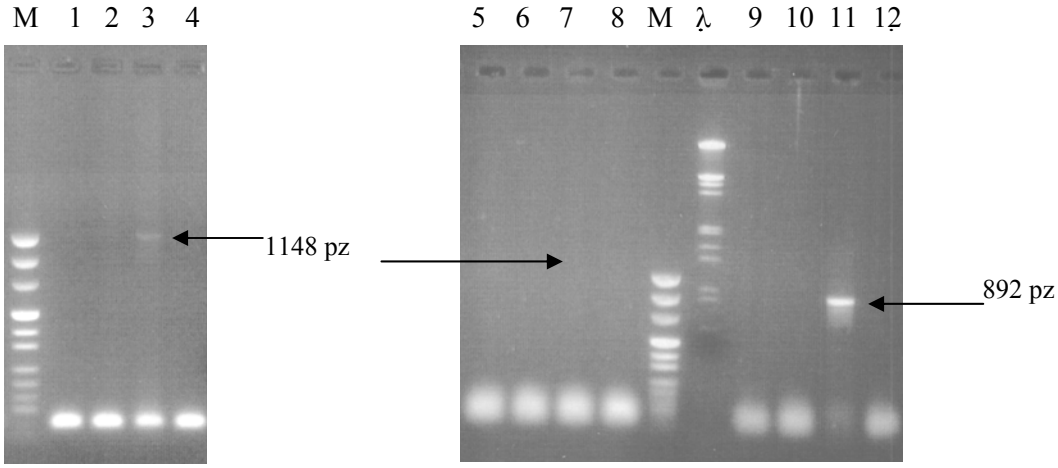


Fig. 2. Species specificity of starters amplifying *ureB Helicobacter felis* gene fragments. Pathway 1, 5 and 9 – negative reagent controls. First stage of nested-PCR reaction with FelF and FelR starters and *Helicobacter heilmannii* DNA (pathway 2 and 6); *Helicobacter felis* (3 and 4) and *Helicobacter pylori* (4 and 8). Second stage of reaction with WfelF and WfelR starters and *Helicobacter heilmannii* DNA (pathway 10); *Helicobacter felis* (11) and *Helicobacter pylori* (12). M – mass marker, pUCMIX8 (Fermentas, SM0301); λ phage digested by *Eco*RI and *Hind*III enzymes (Fermentas SM0191). Product characteristics for subsequent nested-PCR stages are marked with arrows. The photographs show frequently occurring product amplification at first stage, weakly marked in practice. Only at second stage product originates in amount allowing its easy detection.

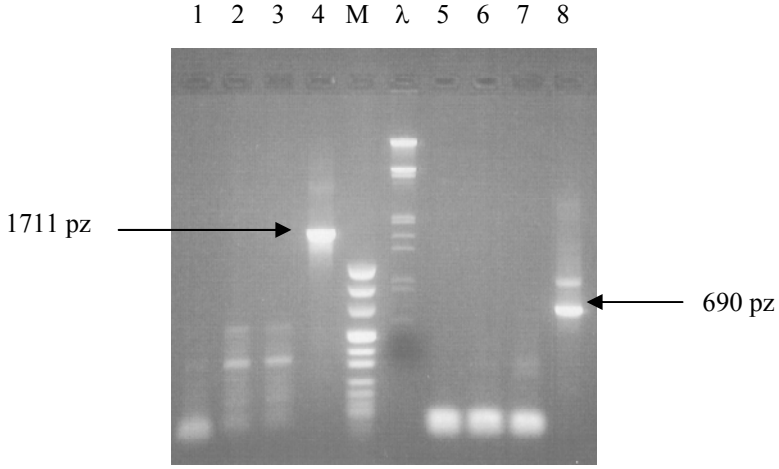


Fig. 3. Species specificity of starters amplifying *ureB Helicobacter pylori* gene fragments. Pathways 1 and 5 – negative reagent controls without DNA. First stage of nested-PCR reaction with PylNF and PylNR starters and *Helicobacter heilmannii* DNA (pathway 2); *Helicobacter felis* (3) and *Helicobacter pylori* (4). Second stage of reaction with WpylF and WpylR starters and *Helicobacter heilmannii* DNA (pathway 6); *Helicobacter felis* (7) and *Helicobacter pylori* (8). M – mass marker, pUCMIX8 (Fermentas, SM0301); λ phage digested by *Eco*RI and *Hind*III enzymes (Fermentas SM0191). Product characteristic for subsequent nested-PCR stages are marked with arrows.

In the nested-PCR method applied for detection of *ureB* genes encoding urease in *Helicobacter heilmannii*, *Helicobacter pylori* and *Helicobacter felis* the positive results were obtained:

- for *Helicobacter heilmannii* – in 114 DNA preparations obtained from dogs' gastric mucosa bioptates,

- for *Helicobacter pylori* – in 7 DNA preparations obtained from dogs' gastric mucosa bioptates,
- for *Helicobacter felis* – in 69 DNA preparations obtained from dogs' gastric mucosa bioptates.

This method gave negative results in 11 DNA preparations obtained from dogs' gastric mucosa bioptates.

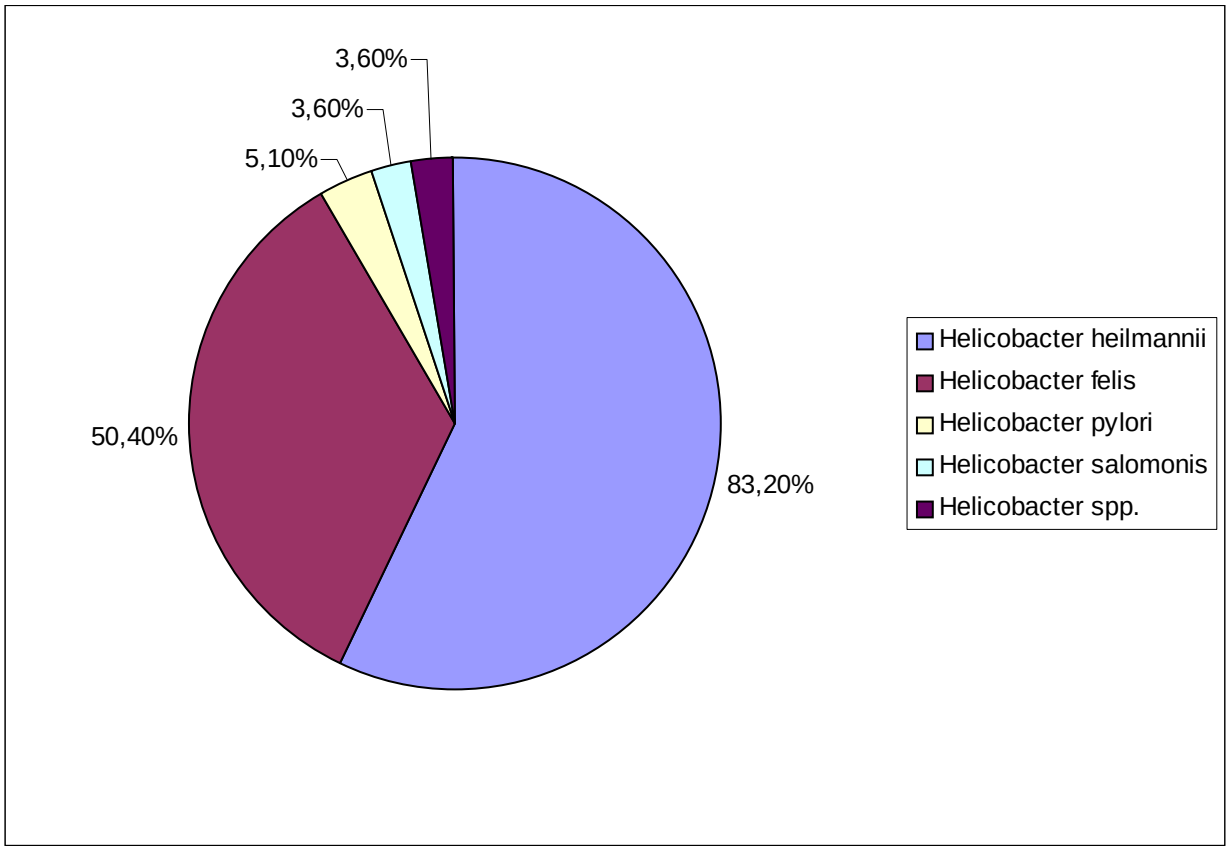
The classical-PCR method with CAR577F and CAR636R starters served to examine 11 DNA preparations from dogs' gastric mucosa bioptates which gave negative results for *ureB* genes. The positive results in the reaction with the above mentioned starters were obtained in 2 DNA preparations from dogs' gastric mucosa bioptates and negative results – in 9 preparations.

The classical-PCR method with Bi1F and Bi2R (*Helicobacter bizzozeronii*) starters and T3B and HT135R (*Helicobacter salomonis*) starters was used to examine 128 DNA preparations from dogs' gastric mucosa bioptates which gave a positive result in the nested-PCR method applied for detection of *ureB* genes encoding urease in *Helicobacter heilmannii*, *Helicobacter pylori*, *Helicobacter felis* and in the classical-PCR method with CAR577F and CAR636R starters. The positive results of the reaction with Bi1F and Bi2R starters and T3B and HT135R starters were obtained only for *Helicobacter salomonis* – 5 DNA preparations from dogs' gastric mucosa bioptates.

Out of 9 DNA preparations from dogs' gastric mucosa bioptates which gave a negative result in the nested-PCR method used for detection of *ureB* genes encoding urease in *Helicobacter heilmannii*, *Helicobacter pylori*, *Helicobacter felis* and in the classical-PCR method with CAR577F and CAR636R starters, seminested-PCR method with HelF, HeilR1 and HeilR2 starters, *Helicobacter* spp. infection was observed in 5 DNA preparations from dogs' gastric mucosa bioptates.

Based on the conducted research *Helicobacter* microorganisms were found in gastric mucosa specimens in 133 dogs. Seventy three dogs had infection with one *Helicobacter* species, i. e.: *Helicobacter heilmannii* was observed in 55 dogs, *Helicobacter felis* – in 11 dogs, *Helicobacter salomonis* – in 2 dogs and unidentified species – in 5 dogs. Fifty three dogs revealed infection with two *Helicobacter* species, in the following patterns: *Helicobacter heilmanni* + *helicobacter felis* – 50 dogs, *Helicobacter heilmanni* + *Helicobacter pylori* – 2 dogs, *Helicobacter felis* + *Helicobacter salomonis* – 1 dog. Six dogs had infection with three *Helicobacter* species, in the following patterns: *Helicobacter heilmannii* + *Helicobacter pylori* + *Helicobacter felis* – 5 dogs, *Helicobacter heilmannii* + *Helicobacter felis* + *Helicobacter salomonis* – 1 dog. One dog suffered infection with four *Helicobacter* species, in the pattern: *Helicobacter heilmannii* + *Helicobacter pylori* + *Helicobacter felis* + *Helicobacter salomonis*. Based on PCR examination 4 definite *Helicobacter* species were observed in the dogs. *Helicobacter heilmannii* was detected in 114 dogs, *Helicobacter felis* – in 69 dogs, *Helicobacter pylori* – in 7 dogs and *Helicobacter salomonis* – in 5 dogs. In 5 animals the PCR reaction result indicated only infection with *Helicobacter* spp. (Fig. 4.).

Fig. 4. Frequency of particular Helicobacter microorganisms occurrence in dogs.



DiscussionA wide range of molecular research applying polimerase chain reaction (PCR), including both the classical-PCR method, nested-PCR and seminested-PCR method used in our study, allowed identification of the following *Helicobacter* species: *Helicobacter heilmannii*, *Helicobacter pylori*, *Helicobacter felis* and *Helicobacter salomonis*. Based on detection of *ureB* genes encoding urease in DNA isolated from the tissues, it was possible to determine if the animals were infected with: *Helicobacter heilmannii*, *Helicobacter pylori* and *Helicobacter felis*. Production of the urease is a diagnostic feature for *Helicobacter* microorganisms. Genes encoding this enzyme, despite considerable conservatism of nucleotide sequences, differ to the degree which may be used as a species specific diagnostic feature [19, 29].

However, in PCR reactions with CAR577F and CAR636R starters specific for 16S rRNA of 3 species, i.e. *Helicobacter bizzozeronii*, *Helicobacter felis* and *Helicobacter salomonis,* which is confirmed by observations of De Groote et al. [6] and next in PCR reactions with Bi1F, Bi2R and T3B, HT135R it was possible to detect *Helicobacter bizzozeronii* and *Helicobacter salomonis* in the examined material [3].

In the case of the animals in which the above mentioned PCR examinations gave negative results, it was assumed that the animals were not infected by microorganisms of *Helicobacter* spp., or they were infected by other species than the mentioned above ones. The conduction of seminested-PCR with He1F, He1R1 and He1R2 starters allowed verification of the above assumption, since applied starters amplify highly conservative 16S rRNA sequence of 251 pz in length which is present in all known *Helicobacter* spp. [33]. The positive test result proved that

the examined material contained *Helicobacter* spp. microorganisms, yet it did not determine its species classification.

The PCR method allowed detection of the following *Helicobacter* species which colonized gastric mucosa in the examined dogs: *Helicobacter heilmannii* (114/137), *Helicobacter felis* (69/137), *Helicobacter pylori* (7/137) and *Helicobacter salomonis* (5/137). The gastric mucosa bioptates of 5 dogs contained *Helicobacter* spp., yet their species was not determined. Only 4 out of 137 animals were free from infection. Hwang et al. [15] detected infection with *Helicobacter heilmannii* in 32 cases out of the total number of 42 animals subject to examination. Only 2 dogs had infection with *Helicobacter feli*. Hwang's et al. [15] observation are similar to ours and confirm the thesis that *Helicobacter heilmannii* is the most commonly observed in the dogs' gastric mucosa. Yet, in relation to infection with *Helicobacter felis* our and their observations differ – in respect to the frequency of occurence of these bacteria: in Hwang's et al. [15] study – 4.8% of dogs, in our study – 50.4 % of dogs. Also Neiger's et al. [20] results are worth noting, as they demonstrated more common infection with *Helicobacter bizzozeronii*/*Helicobacter salomonis* in dogs than with *Helicobacter felis*.

Our studies, based on PCR, showed the presence of *Helicobacter pylori* in the gastric mucosa bioptates in 8 dogs. In medical literature authors state that this microorganism may colonize gastric mucosa in cats and has not been found in dogs so far [5, 7, 9, 12, 24]

C O N C L U S I O N S

1. The combined application of classical-PCR, nested-PCR and seminested-PCR using appropriate primers allows the best species idetification of *Helicobacter* microorganisms, found in the dogs' stomach.
2. The infection of the gastric mucosa with *Helicobacter* species is common in dogs.
3. The most common species colonizing the gastric mucosa in dogs is *Helicobacter heilmannii*.
4. The dogs' gastric mucosa may be colonized by *Helicobacter pylori*.

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ВИКОРИСТАННЯ ПОЛІМЕРНОЇ ЛАНЦЮГОВОЇ РЕАКЦІЇ (ПЛР), ГНІЗДОВОЇ ПЛР ТА НАНІВГНІЗДОВОЇ ПЛР У ІДЕНТИФІКАЦІЇ ВИДІВ *HELICOBACTER,* ЯКІ СТВОРЮЮТЬ КОЛОНІЇ У СЛИЗОВІЙ ОБОЛОНЦІ ШЛУНКА СОБАК ПРИ ГАСТРИТІ

Р е з ю м е

1. Комбіноване застосування класичного ПЛР, гніздового-ПЛР, напівгніздового-ПЛР, з використанням відповідних капсул дозволяє ефективно ідентифікувати види мікроорганізмів *Helicobacter* у шлунку собак.

2. Інфікування слизової оболонки шлунка видами *Helicobacter* є поширеним у собак.

3. Найпоширенішими видами, які створюють колонії в слизовій оболонці шлунка собак є *Helicobacter heilmannii*.

4. У слизовій оболонці шлунка собак можуть утворюватися колонії *Helicobacter pylori.*

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ИСПОЛЬЗОВАНИЕ ПОЛИМЕРНОЙ ЦЕПНОЙ РЕАКЦИИ (ПЦР), ГНЕЗДОВОЙ ПЦР И ПОЛУГНЕЗДОВОЙ ПЦР В ИДЕНТИФИКАЦИИ ВИДОВ *HELICOBACTER,* КОТОРІЕ ОБРАЗУ ЮТ КОЛОНИИ В СЛИЗИСТОЙ ОБОЛОЧКЕ ЖЕЛУДКА СОБАК ПРИ ГАСТРИТЕ

А н н о т а ц и я

1. Комбинированное применение классического ПЦР, гнездового ПЦР, полугнездового PCR с использованием соответствующих капсул позволяет эффективно идентифицировать виды микорооганизмов *Helicobacte* в желудке собак.

2. Инфицирование слизистой оболочки желудка видами *Helicobacte* распространено у собак.

3. Самыми распространенными видами, которые создают колонии в слизистой оболочке собак являются *Helicobacter heilmannii.*

4. В слизистой оболочке желудка собак могут создавать колонии Helicobacter pylori.

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