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Сравнение методов диагностики *H.pylori* в биоптатах желудка при различной патологии гастроинтестинального тракта

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Comparison of methods for the diagnosis of *H.pylori* in gastric biopsy specimens from patients with different gastrointestinal diseases

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Аннотация

В данной работе представлено сравнение микробиологического и экспресс-теста на уреазу для диагностики хеликобактериоза с методом ПЦР в биоптатах от пациентов с диагнозами гастродуоденит, хронический гастрит, бульбит и рефлюкс-эзофагит. В общем был получен тренд на большее количество ложноположительных результатов при использовании экспресс-метода и ложноотрицательных при использовании микробиологического, однако статистически достоверной данная закономерность являлась только для диагнозов гастродуоденит и хронический гастрит. Была построена прогностическая модель для определения влияния метода исследования на результат.

Ключевые слова

***H.pylori*, хеликобактер, сравнение методов.**

Summary

This paper presents a comparison of cultural and rapid urease tests for the detection of *H.pylori* presence with PCR in biopsy specimens from patients diagnosed with gastroduodenitis, chronic gastritis, duodenitis and reflux oesophagitis. In general, there was a trend towards more false positive results when using the rapid urease testing method and false negatives when using the cultural method is used, but this pattern was statistically significant only for the diagnoses of gastroduodenitis (for all methods) and chronic gastritis (there was no statistically significant difference between PCR and the cultural method). A prognostic model was constructed to determine the effect of the test method on the outcome.

Keywords

***H.pylori*, *Helicobacter pylori*, diagnostic methods.**

Introduction

Helicobacter pylori was discovered almost 40 years ago and its role in the development of stomach diseases has been proven in a lot of studies. Since then, a wide variety of methods has been developed to establish the presence of *H.pylori* in the human body. Traditionally those methods are divided into invasive and non-invasive.

Invasive methods use gastric biopsy specimens to detect *H.pylori*. These methods include the cytological method, rapid urease test and cultural methods. Apart from direct detection of *Helicobacter* in the specimen using a microscope and different staining methods, histology is a great way to assess the inflammation and associated pathology like metaplasia or cancerous lesions. However, this method is rather expensive and dependent on different bacterial densities at different sites [1, 2]. Another popular invasive method is the rapid urease test. Usually, a biopsy specimen is placed in the test system containing urea and a certain dye that changes its colour depending on the acidity of the media. If the specimen has high concentrations of *H.pylori*, the indicator changes colour. Some advantages of this method include simplicity and a short time to get the results. However, this method does not detect the direct presence of *H.pylori* in

the biopsies. There is evidence that several other microbes can produce urease, which can affect the frequency of false-positive results of such a test. And in the case of urease-negative *H.pylori* strains, this test can give false-negative results [3–5]. Last but not least, the cultural method has the highest specificity in this group and can be used to establish if a strain has certain pathogenic factors (eg enzymes) as well as to establish the susceptibility to antibiotics. However, this method is slow and pricey. Moreover, it has a lower sensitivity compared to other methods, partially because *H.pylori* is quite fastidious about every aspect of cultivation from transportation to the atmosphere and culture media [6–9].

Non-invasive methods include urea breath test and serology. Because of its speed and rather high sensitivity and specificity, the urea breath test is considered to be one of the best non-invasive methods for *H.pylori* detection. It measures the activity of *H.pylori* urease detecting a ^{13}C or ^{14}C isotope in the breath of a patient after she drinks a solution with the labelled isotope. However, this test is rather expensive and requires sophisticated equipment. Moreover, there is a number of scenarios where it can give a false-positive result [7, 10, 11]. Serology is a method that detects antibodies to *H.pylori* in patient biological fluids, such as blood, blood serum, saliva or urine. The most commonly used ways to detect those antibodies are enzyme-linked immunosorbent assay, western blotting and enzyme immunoassays. However, there is evidence that Immunoglobulin (Ig) M tests for *H.pylori* infection have little diagnostic value. As for IgG, which is the most reliable antibody to test for, there is a delay in the onset of *H.pylori* infection and the start of their production. That makes it difficult to differentiate between the active infection and previous exposure to the pathogen [7, 12].

There are also molecular methods of *H.pylori* detection which can be attributed to a separate group. One of the most used methods in this group is polymerase chain reaction (PCR). When biopsy specimens are used to detect *H.pylori* DNA, this molecular method can be attributed to this group. If PCR uses stool samples or other biological material that does not require invasive manipulations to the human body to detect the presence of *H.pylori* DNA, then it can be included in the non-invasive methods. Molecular methods are highly specific and sensitive, but their use in clinical practice is limited by the high price and complexity [13–15].

Each of the methods above has its limitations and benefits, but up to this day, there is no single gold standard in diagnostics of *H.pylori* infection in humans universally agreed upon by the researchers [16, 17]. This article aims to expand the current knowledge of some diagnostic methods for *H.pylori* detection with new data.

Goal. This study aimed to compare the cultural and rapid urease methods of identifying *Helicobacter pylori* in gastric biopsies with the PCR and study the differences in these methods in different gastric diseases.

Materials and methods

A total of 137 biopsy specimens of gastric mucosa, taken from 137 patients with chronic diseases of the gastrointestinal tract were studied. 27 patients had chronic gastritis (ICD-10 K29.3, K29.4, K29.5), 69 patients had gastroduodenitis (ICD-10 K29.9), 17 patients had duodenitis (ICD-10 K29.8), 24 patients had gastro-oesophageal reflux disease with esophagitis (reflux oesophagitis) (ICD-10 K21.0). The patients were aged 21 to 74 years with a mean age of 38. Out of those patients, 75 were women and 63 were men. Biopsy samples were collected at the Samara Diagnostic Centre between December 2015 and February 2017.

Each biopsy specimen was collected following the recommendations of the V Maastricht Consensus [10]. Four biopsy specimens were taken from each patient. One of the biopsy specimens was used to perform a rapid urease test using the HELPIL® system (Association of Medicine and Analytics LLC, Russia), the others were placed in sterile tubes with thioglycolic medium (SSC PMB Obolensk, Russia) and within a one-hour timeframe transported to the Samara State Medical University Clinical Lab. There a real-time PCR test was performed on a CFX 96 Touch real-time PCR machine (Bio-Rad, USA) using HELICOPOL-RV (NPF-Litech, Russia) reagent kit.

In addition to the PCR test, *H.pylori* was cultivated using cultural mehthod. The results of the *H.pylori* indication, isolation and identification study were analysed and compared. Selective and non-selective media were used for the cultivation of *H.pylori*. The selective medium was Colombian agar with the addition of sheep red blood cells and a selective mixture of antibiotics: 5 mg vancomycin, 2.5 mg trimethoprim, 2.5 mg cefsulodin, 2.5 mg amphotericin B (HiMedia Laboratories, India), non-selective was Colombian agar with the addition of sheep red blood cells without selective additive (HiMedia Laboratories, India). Incubation was carried out in anaerobic jars. A microaerophilic atmosphere (oxygen concentration between 5-14% and carbon dioxide concentration between 10-15%, as *Helicobacter* is a capnophile) was created using GenBag Microaero commercial gas generating packages (Biomerieux, France). The results were

evaluated on day 4-5. All grown colonies were subjected to identification procedures.

Colonies with culture properties characteristic of *H. pylori* were subjected to a biochemical identification procedure. Colony selection was performed according to the following features: small, round, smooth, transparent, «dewdrop-like» colonies, up to 3 mm in diameter (1 mm on average) on nutrient media.

Identification was based on the characteristic microscopic picture, cultural and biochemical properties. Three tests were performed for biochemical identification. The urease test was performed with a 2% urea solution with phenol red indicator (LenReactiv, Russia) in Eppendorf-type tubes. The oxidase test was performed using commercially available test strips (Erba Lachema, Czech Republic). The catalase test was performed on glass by introducing a pure *Helicobacter* culture in a drop of 10% hydrogen peroxide solution (Micro-CATALAZA-NICP kit, Research Center of Pharmacotherapy, Russia). *H.pylori* identification was considered acceptable if all positive results for the above indications were obtained.

Statistical analysis was performed using StatTech v. 2.4.3 (StatTech LLC, Russia). The results were analysed taking into account the diagnosis of the patients. Grouping of primary data was performed using Microsoft Exel® 2013 software package. Categorical data were described with absolute values and percentages. Comparison of percentages in multifield contingency table analyses was performed using Pearson's X^2 test to estimate the degree of event regularity (p). The association between features was statistically evaluated as significant at a significance level of $p<0.001$ or $p<0.005$ depending on test statistics.

Predictive modelling of the probability of a particular outcome was performed using logistic regression. Nagelkerke's R^2 was used as a measure of certainty, indicating that part of the variance could be explained by the logistic regression. The significance of the regression model was assessed at $p<0.005$.

Results and discussion

There were no statistically significant differences in the sex and age pattern between the patients. Patients with isolated forms of gastric and duodenal mucosal lesions are characterised by a considerable variety of data on the evaluation of *H.pylori* involvement in the pathological process. To identify potential reasons for significant discrepancies, different methods of determining *H.pylori* in biopsy material from patients with chronic gastric and duodenal disease were compared.

In line with the trend in recent years, PCR was chosen as the reference method among *Helicobacter* researchers. High sensitivity and specificity of PCR for the detection of *H.pylori* DNA in biopsy material were determining factors in the choice of the reference method [7, 17, 18].

PCR detected the presence of *H.pylori's* DNA in 103 out of 137 samples, representing 75.2% of all samples. Analysis of the frequency of *H.pylori* DNA detection in biopsy material depending on the diagnosis yielded results ranging from 62.5% in gastro-oesophageal reflux disease with oesophagitis to 82.4% in duodenitis. It should be noted there was no statistically significant difference between the frequency of *H.pylori* detection by PCR depending on diagnosis in patients with gastroduodenitis and reflux oesophagitis (p=0.129), gastroduodenitis and chronic gastritis (p=0.661), gastroduodenitis and duodenitis (p=0.711), reflux oesophagitis and chronic gastritis (p=0.374), reflux oesophagitis and duodenitis (p=0.169), chronic gastritis and duodenitis (p=0.524). The results of *H.pylori* DNA detection in biopsy specimens by PCR depending on the diagnosis are shown in Table 1.

To sum up, the frequency of *H.pylori* DNA detection in biopsy specimens did not depend on the diagnosis of the patient. This is another factor why PCR is a good measure to compare different methods of *H.pylori* detection in gastric biopsy.

As the second step, the biopsy material from all patients was examined with the cultural method to isolate a pure culture of *H.pylori*. Comparing the frequency of *H.pylori* isolation from biopsy specimens with the PCR, a significantly lower detection rate was observed. Out of 137 patients, *H.pylori* was cultured in only 56.9% of cases. As in the PCR study, with the cultural method there was no statistically significant difference in the *H.pylori* detection frequency depending on the diagnosis of the patients (in all cases $p>0.05$). Thus, whilst using a cultural method, there was no statistically significant differences between different gastric diseases in terms of *H.pylori* detection rates. The results of *H.pylori* isolation in biopsy samples are presented in Table 2.

When the results of urease activity were analysed as a rapid test as a proxy for the detection of *H.pylori* in the biopsy specimens, the following data was obtained. The proportion of positive results was 88.3% overall. The lowest results were shown in reflux oesophagitis at 66.7% and the highest in chronic gastritis at 96.3%.

Statistical processing of the obtained results also revealed no statistically significant differences in the frequency of positive results of rapid urease activity test depending on the disease in

Table 1. *Helicobacter pylori* DNA detection frequency in biopsy samples by PCR depending on the diagnosis

Diagnosis	Number of positive results, (n)	Total number of the patients with this diagnosis, (n)	Proportion of the positive results from the total, (%)
Gastroduodenitis	54	69	78,3%
Reflux Oesophagitis	15	24	62,5%
Chronic Gastritis	20	27	74,1%
Duodenitis	14	17	82,4%
Total	103	137	75,2%

Table 2. Results of *Helicobacter pylori* cultivation from biopsy specimens

Diagnosis	Number of positive results, (n)	Total number of the patients with this diagnosis, (n)	Proportion of the positive results from the total, (%)
Gastroduodenitis	38	69	55,1%
Reflux Oesophagitis	14	24	58,3%
Chronic Gastritis	14	27	51,9%
Duodenitis	12	17	70,6%
Total	78	137	56,9%

comparing results of patients with gastroduodenitis and chronic gastritis (p=0,398), gastroduodenitis and duodenitis (p=0,704), chronic gastritis and duodenitis (p=0,739). At the same time, statistically significant differences were revealed in the results of the rapid urease activity test when comparing data in patients with reflux oesophagitis and other diseases: reflux oesophagitis and gastroduodenitis (p=0,004), reflux oesophagitis and chronic gastritis (p=0,006), reflux oesophagitis and duodenitis (p=0,037). In all cases, the proportion of positive results in reflux oesophagitis was significantly lower than in other diseases. The results of rapid tests for urease activity in biopsy specimens depending on the disease are shown in Table 3.

Since the urease test has low specificity and gives a large number of false positives, the excessive positive results may be explained by the microflora colonising the gastric mucosa, which is known to have the ability to produce urease. At the same time, the microflora colonising the mucosa in reflux oesophagitis may not have these properties. To identify additional patterns, it was decided to perform a comparative analysis between the results of the different diagnostic methods depending on the diagnosis, calculating Pearson's X^2 and determining a significance level of p.

The results of the overall incidence of a positive result in all patient groups are presented in Table 4.

Statistically significant differences were found when comparing all three groups of methods, as well as when comparing the results of the cultural method with PCR and the rapid urease test. This confirms the need for a more detailed analysis of the impact of the results of the analysed methods depending on the disease, especially considering the significant differences obtained in the first part of the study when comparing the urease activity score in the different patient groups.

The results of this analysis can be seen in Table 5. For all of the gastric diseases, a similar trend was seen. When a rapid urease test is used to establish *H.pylori* presence in the biopsy specimens, there was a tendency to have a false-positive result. On the other hand, using the cultural method for the same purpose had an increased number of false-negative results. However, this trend was proven to be statistically significant between all the three methods only in gastroduodenitis and between the two methods in chronic gastritis (there was no statistically significant difference between the cultural method and PCR).

To confirm the hypothesis about the influence of the *H.pylori* identification method on the positive or negative result depending on the disease, a predictive model for each diagnosis was developed using binary logistic regression in addition to calculating Pearson's X^2 for the obtained data.

The observed dependence for each individual diagnosis was described by an equation:

$$P=1/(1+e^{-z})\times 100\%$$

The prognostic regression model for all of the diagnoses was calculated according to the equation described above.

Table 3. Results of rapid urease tests in biopsy specimens

Diagnosis	Number of positive results, (n)	Total number of the patients with this diagnosis, (n)	Proportion of the positive results from the total, (%)
Gastroduodenitis	63	69	91,3%
Reflux Oesophagitis	16	24	66,7%
Chronic Gastritis	26	27	96,3%
Duodenitis	16	17	94,1%
Total	121	137	88,3%

Table 4. Influence of the *Helicobacter pylori* diagnostic methods on the test results

Result	Method			p
	Cultural, n (%)	PCR, n (%)	Rapid Urease Test, n (%)	
	1	2	3	
Positive	59 (43,1)	34 (24,8)	16 (11,7)	< 0,001*
Negative	78 (56,9)	103 (75,2)	121 (88,3)	1vs2=0,003
				1vs3<0,001
				2vs3=0,005

* – differences are statistically significant (p<0,05)

Table 5. Results of *H.pylori* detection methods in patients with different diagnoses

Diagnosis	Result	Method			p
		Cultural,n (%)	PCR, n (%)	Rapid Urease Test, n (%)	
Reflux	Negative	10 (41,7)	9 (37,5)	8 (33,3)	0,837
Oesophagitis	Positive	14 (58,3)	15 (62,5)	16 (66,7)	
Duodenitis	Negative	5 (29,4)	3 (17,6)	1 (5,9)	0,198
	Positive	12 (70,6)	14 (82,4)	16 (94,1)	
Gastroduodenitis	Negative	31 (44,9)	15 (21,7)	6 (8,7)	<0,001*
	Positive	38 (55,1)	54 (78,3)	63 (91,3)	1vs2=0,008
					1vs3<0,001
					2vs3=0,033
Chronic Gastritis	Negative	13 (48,1)	7 (25,9)	1 (3,7)	<0,001*
	Positive	14 (51,9)	20 (74,1)	26 (96,3)	1vs2<0,001
					2vs3=0,043

* – differences are statistically significant (p<0,05)

For gastroduodenitis

$$1{,}281+1{,}070X_{\text{Rapid urease test}}-1{,}077X_{\text{Cultural}}.$$

For chronic gastritis

$$z=1{,}050+2{,}208X_{\text{Rapid urease test}}-0{,}976X_{\text{Cultural}}.$$

For reflux oesophagitis

$$z=0{,}511+0{,}182X_{\text{Rapid urease test}}-0{,}174X_{\text{Cultural}}.$$

For duodentitis

$$z=1{,}540+1{,}232X_{\text{Rapid urease test}}-0{,}665X_{\text{Cultural}}.$$

Where P is the probability of a positive result, $X_{\text{Rapid urease test}}$ – Method (0 – PCR, 1 – Rapid urease test), X_{Cultural} – Method (0 – PCR, 1 – Cultural).

The calculated regression model was statistically significant (p<0.001) for gastroduodenitis and chronic gastritis. From the Nagelkerke determination coefficient value, the model explains 17.1% in gastroduodenitis and 26.1% in chronic gastritis of the observed variance of the outcome measures. When assessing the impact of the method on the probability of a result, the use of the rapid method to detect *H.pylori* in biopsy specimens obtained from patients with relevant diagnoses was accompanied by an increase in the probability of a positive result. In contrast, the use of the microbiological method in the impact assessment was accompanied by a decrease in the probability of a positive result.

In these diseases, the use of the rapid urease test was accompanied by a higher probability of a positive result. This corresponds with the data that apart from

H.pylori there is urease-positive flora in the stomach that may often give false positives in the rapid urease test of the biopsy specimens. However, there is very little evidence in the literature as to why positive results may be reduced when choosing a cultural method for these two diseases.

When assessing the effect of the method on the outcome for duodenitis and reflux oesophagitis, the rapid method was accompanied by an increase in the probability of a positive result and the cultural method has seen a decrease in the probability of a positive result. However, the regression model for these diagnoses was not statistically significant (duodenitis: p=0.175; reflux oesophagitis: p=0.837). Based on the Nagelkerke determination coefficient, the model for duodenitis explained only 10.9% of the observed variance in the outcome measure, while the model for reflux oesophagitis explained 0.7%.

Thus, the choice of method to detect *Helicobacter* in the biopsy specimen for reflux oesophagitis and duodenitis does not affect the likelihood of a positive result. This means that, despite the tendency for a lower number of positive results with microbiological testing, only PCR testing should be used as a diagnostic method for *H.pylori* detection in these diseases.

Conclusion

The data presented in this article shows that despite the diagnoses, there is a common trend of detecting *H.pylori* in biopsy specimens. In comparison with the PCR method, the rapid urease test is more likely to give false-positive results, while the cultural method is more likely to give false-negative results. This requires a deeper analysis and identification of the possible influence of microenvironmental factors of *H.pylori* environment in chronic inflammation of different localizations. While the urease-positive microflora more prevalent in the stomach may explain the false-positive results in all cases except reflux oesophagitis, the significantly lower probability of obtaining a pure *H.pylori* culture with the cultural method may be caused by the contaminating microflora, which may have a negative effect when co-cultured with *H.pylori* using both selective and conventional artificial media. Further research is needed to prove or disprove this hypothesis.

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