FIRST CONFIRMATION OF *BORDETELLA PERTUSSIS* OCCURRENCE IN SLOVAKIA BY USING REAL-TIME PCR

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SUMMARY

By application of the real-time PCR we manage to confirm the diagnosis and occurrence of a disease, which is caused by *Bordetella pertussis* – pertussis. Using this method we have proven the presence of DNA of *Bordetella pertussis* in the biological materials (nasopharyngeal swabs). The presence of IS481 genome sequence of *Bordetella pertussis* was confirmed. This method of detection of pathogens seems to be very rapid, simple, and specific. In the case of adequate technical laboratory equipment it may become very suitable and important supporter in explanation and confirmation of the occurrence of bacterial infections.

Key words: Bordetella pertussis, whooping cough, real-time PCR, LightCycler

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INTRODUCTION

The application of knowledge of bacterial genetics is no longer a new issue and the domain of the research and development centres, but they are increasingly applied in microbiological practice. Using molecular-biology methods it is possible to confirm the clinical diagnosis, where other detection methods have failed.

Definitive diagnosis of causative organism in pertussis (whooping cough), *Bordetella pertussis*, is traditionally been made by culture of secretions from the nasopharynx (1).

Serological tests like CFT (Complement Fixation Test) or ELISA (Enzyne-linked Immunosorbent Assay) (detection of antibodies produced against a filamentous hemaglutinin or against pertussis toxin) are more sensitive than culture, but these usually provide late, or retrospective diagnosis only (2).

To overcome these limitations, detection of *B. pertussis* DNA from nasopharyngeal aspirates and swabs has been described using PCR assays (3, 4, 5, 6, 7, 8).

In 2001 Kösters at al. have developed a real-time PCR assay based on the TaqMan technology for detection of *Bordetella pertussis* in clinical samples (9). One year before Reischel et al. have described a real-time PCR technique for detection of *Bordetella pertussis* by using LightCycler instrument and fluorescent labelled probes (10). We use this method in our department for diagnosis of pertussis.

MATERIALS AND METHODS

Material

Samples: From December 2004 to the February 2005 33 nasopharyngeal swabs – taken from 29 patients from Nitra and Topoľčany, were examined. Dacron tampons were used because previous studies had shown that calcium alginate fiber and aluminium shaft components inhibited PCR reaction (11). Materials were delivered to the laboratory by a special courier.

First case suspected of *Bordetella pertussis* infection was a little boy from Nitra (Table 1) Nasopharyngeal swab was taken by epidemiologist from this boy, because the clinical symptoms indicated the presence of *Bordetella pertussis*. Next three swabs were taken from his brother (Table1) and two sisters, neighbours of these two boys (Table1). From one of these sisters there was taken next one swab with off-set (Table 1).

Table 1. *Results of sample examination for B. pertussis – Nitra 2004*

Number of sample	Date of birth	Date of samples taking	Date of examination	Result
1/04	01.05. 2004	08. 12. 2004	15. 12. 2004	POSITIVE
2/04	20. 12. 1990	08. 12. 2004	15. 12. 2004	NEGATIVE
3/04	12. 09. 1985	23. 12. 2004	05. 01. 2005	POSITIVE
4/04*	12. 09. 1985	05. 01. 2005	05. 01. 2005	POSITIVE
5/04	07. 01. 1991	05. 01. 2005	05. 01. 2005	POSITIVE

* repeated taking of sample with off-set

Table 2. *Results of sample examination for B. pertussis – Topoľčany 2005*

Number of sample	Date of birth	Date of samples taking	Date of examination	Result
3/05	26. 11. 2004	20. 01. 2005	21. 01. 2005	POSITIVE
4/05	03. 04. 1981	31. 01. 2005	02. 02. 2005	POSITIVE
5/05	16. 04. 1942	31. 01. 2005	02. 02. 2005	POSITIVE
8/05	04. 08. 1993	31. 01. 2005	02. 02. 2005	NEGATIVE
9/05	22. 05. 1995	31. 01. 2005	02. 02. 2005	POSITIVE
10/05	15. 03. 1978	31. 01. 2005	02. 02. 2005	NEGATIVE
11/05	11. 12. 1982	31. 01. 2005	02. 02. 2005	NEGATIVE
12/05	15. 04. 2003	31. 01. 2005	02. 02. 2005	NEGATIVE
13/05	30. 09. 1954	31. 01. 2005	02. 02. 2005	NEGATIVE
14/05	05. 07. 1951	31. 01. 2005	02. 02. 2005	POSITIVE
15/05	06. 10. 1981	31. 01. 2005	02. 02. 2005	NEGATIVE
16/05	08. 06. 1976	31. 01. 2005	02. 02. 2005	POSITIVE
17/05	01. 03. 1973	31. 01. 2005	02. 02. 2005	NEGATIVE
18/05	16. 05. 2001	31. 01. 2005	02. 02. 2005	NEGATIVE
19/05	09. 01. 1983	31. 01. 2005	02. 02. 2005	NEGATIVE
20/05	25. 12. 2004	31. 01. 2005	02. 02. 2005	NEGATIVE
21/05*	26. 11. 2004	31. 01. 2005	02. 02. 2005	NEGATIVE
22/05	24. 01. 2000	31. 01. 2005	02. 02. 2005	NEGATIVE
23/05	09. 02. 2004	31. 01. 2005	02. 02. 2005	NEGATIVE
24/05	04. 09. 1962	31. 01. 2005	02. 02. 2005	NEGATIVE
25/05	17. 08 1978	31. 01. 2005	02. 02. 2005	NEGATIVE
26/05	28. 05. 1946	31. 01. 2005	02. 02. 2005	DISPUTE

* repeated taking of sample with off-set

In January 2005 we have obtained a nasopharyngeal swab from a little boy who lived in Topoľčany (Table 2). When epidemiologists came to check the home of this boy and his mother, they found next to 20 people in bad hygienic conditions. There were 9 people in two-room flat and 11 people in one-room flat. Therefore they took 20 nasopharyngeal swabs from them (Table 2), one repeated swab from a little boy (Table 2) and also two control swabs from themselves. These swabs we used as negative control of nucleic acid isolation (Table 3). At the time of taking samples epidemiologists did not have any clinical symptoms of pertussis.

Table 3. *Control nasopharyngeal swabs from two epidemiologists, negative control of nucleic acid isolation*

Number of sample	Date of birth	Date of samples taking	Date of examination	Result
6/05	30. 06. 1970	31. 01. 2005	02. 02. 2005	NEGATIVE
7/05	30. 10. 1962	31. 01. 2005	02. 02. 2005	NEGATIVE

Table 4. *Topoľčany 2005, later taking of samples*

Number of sample	Date of birth	Date of samples taking	Date of examination	Result
28/05	11. 12. 1993	15. 02. 2005	01. 03. 2005	NEGATIVE
29/05*	25. 12. 2004	15. 02. 2005	01. 03. 2005	NEGATIVE
30/05*	15. 04. 2003	15. 02. 2005	01. 03. 2005	NEGATIVE
31/05	11. 04. 1956	15. 02. 2005	01. 03. 2005	NEGATIVE
32/05	22. 12. 1960	15. 02. 2005	01. 03. 2005	NEGATIVE
33/05	07. 02. 2004	15. 02. 2005	01. 03. 2005	NEGATIVE

* repeated taking of sample with off-set

One month later epidemiologists from Topoľčany sent us four new nasopharyngeal swabs from other inhabitants of these flats and also two repeated swabs from two children (Table 4).

METHODS

Isolation of DNA from the sample: We have isolated the bacterial DNA using the High Pure PCR Template Kit (12) according to the manufacturer´s protocol. We have used 5 µl of isolated bacterial DNA for each of the reaction.

Two control swabs from epidemiologist were used as negative control of nucleic acid isolation.

Bordetella pertussis strain, CNCTC 5268, from collection of microorganisms, from the National Institute of Public in Prague, was used as positive control of nucleic acid isolation.

PCR: PCR was performed using the LightCycler PCR instrument, LighCycler software version 3.5.3 and the LightCycler Fast Start DNA Master Hybridization Probes Kit (13), all from Roche Diagnostics. The kit contains Taq DNA polymerase, MgCl2 solu-

Table 5. *Primers and probes sequences*

Bordetella pertussis repetitive insertion sequence *IS481*		
	Length	GC (%)
Primers		
GATTCAATAGGTTGTATGCATGGTT	25	36.0
TTCAGGCACACAAACTTGATGGGCG	25	52.0
Probes		
TCGCCAACCCCCCAGTTCACTCA- - F	23	60.9
LCRed640- - AGCCCGGCCGGATGAACACCC	21	71.4

Table 6. *Master mix compositon*

	Volume (µl)	Final concentration
LightCycler fast start DNA master hybridization probes (10x)	2	1 x
$MgCl_2$ stock solution (25 mM)	1.6	3 mM
Primers (10 µM each)	1 + 1	0,5 µM each
Hybridization Probes (2 µM each)	2 + 2	0,2 µM each
PCR- grade water	8.4	
Total volume	18	

tion, PCR-grade water and free nucleotides. Primers and probes were synthetized by TibMol Biol (Berlin, Germany) according to previous study (10). A previously published primer pair (5) and probes (10) (Table 5) were used for amplifying a segment within the *Bordetella pertussis* repetitive insertion sequence *IS481*, which proved to be a very sensitive target (10).

The following master mix was used for amplification and hybridization probe-based detection of the *IS481* specific amplicons (Table 6).

To complete the amplification mixtures, 18 µl of master mix and 2 µl of the corresponding template DNA preparation were added to each capillary. After a short centrifugation (7sec/ 3000 g), the sealed capillaries were placed into the LightCycler rotor.

The presence of *IS481* of BP in clinical specimens was proven under identical reaction conditions, together with positive and negative controls. The reaction ran in the following steps (10,14):

Denaturation 10 min at 95 °C. Amplification was done in 50 cycles. For one cycle there were the following conditions: 10 sec at 95 °C (denaturation), 10 sec at 50 °C (annealing – application of primers and probes), 12 sec at 72 °C (amplification – DNA synthesis).

Control of reaction: At the same time and under the same conditions, the control of procedure of PCR was inspected using the positive and negative controls. The negative control sample was prepared by replacing the DNA template with PCR-grade water. The positive control sample was prepared by adding 2 µl of *Bordetella pertussis* genomic DNA (*Bordetella pertussis* strain, CNCTC 5268, from the National Institute of Public Health in Prague) to the master mix. The genomic DNA from this strain was isolate using High Pure PCR Template Isolation Kit (12), according to the manufacturer´s manual.

Electrophoresis: After the completion of amplification in LightCycler instrument we have analysed products of amplification also by using electrophoresis in agarose gel (90V/ 20 min).

RESULTS AND DISCUSSION

First according to author´s protocol (10), we had to introduce this new diagnostic method for detection of *Bordetella pertussis* into the microbiology laboratory practice. Then this method was tested with reference sample which was the strain of *Bordetella pertussis* (CNCTC 5268, from collection of microorganisms, from the National Institute of Public Health in Prague). In the next step we confirmed the detection limit of this reaction, which was originally done by Reischel et al. (10) and it was 1 pg of DNA template.

We tested 33 nasopharyngeal swabs from 29 patients and 2 control swabs from epidemiologists, which we used as negative controls of nucleic acid isolation.

First laboratory confirmed case of whooping cough was reported from the little boy, born 01. 05. 2004, resident of Nitra. He suffered from a pertussis-like symptoms and the diagnosis was also confirmed because of uncompleted vaccination. First dose of vaccination was applied to him on 30. 08. 2004. He was sick from 02. 09. 2004.

From the beginning the affection had febrile progress (38 °C) with graduated cough followed by vomiting. In the time of incubation period the boy stayed abroad. (Germany, Mannheim – 28. 07. 2004–09. 08. 2004). Doctors treated him by antibiotics for 17 days (Penbene+Zinat).

Epidemiologists found out that brother of this boy, born 20. 12. 1990, was in a contact with him all days. He had clinical symptoms of pertussis for longer time before, but his illness was not laboratory confirmed. His vaccination and revaccination was completed (DITEPER).

Next two laboratory confirmed cases of whooping cough – pertussis were two sisters, neighbours of these two boys (brothers). One of them, born on 07. 01. 1991, was the first case of illness in a family and in the dwelling-place too. Probably she imported the illness from abroad because of her stay in Paris (01. 07. 2004–06. 07. 2004) and in Germany (Mannhiem 07. 07. 2004–08. 07. 2004). Ten days after arriving home she had typical clinical symptoms of pertussis. Her vaccination and revaccination was completed (DITEPER). She was treated by combination of antibiotics (Duracef, Ospamox, Zinnat).

Her sister, born on 12. 09. 1985, was the second case of illness in the family and in the dwelling-place too. This illness was also laboratory confirmed. Her vaccination and revaccination was completed (DITEPER). She was treated by Azitrox.

We did not succeed in obtaining the information on patients from Topolčany and about their vaccination and revaccination status.

Based on the analyses of increasing fluorescent curves at real-time PCR, the presence of *IS481 of Bordetella pertussis* in 22 nasopharyngeal swabs from 20 patients was not confirmed, however, the presence of *Bordetella pertussis* was confirmed in 10 nasopharyngeal swabs from 9 patients (Figs. 1–5, Tables 1–4). In one case the result was disputable, because the fluorescent curve

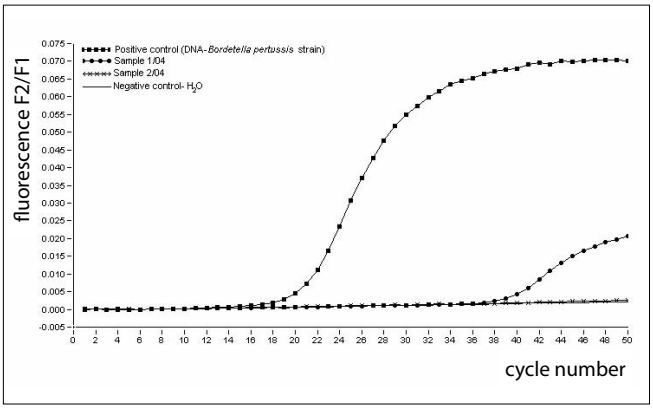

Fig. 1. *Analysis of PCR. With growing number of cycles during amplification, values of fluorescence at some examinated sample (1/04) grow, what suggests presence of B. pertussis in examinated sample.*

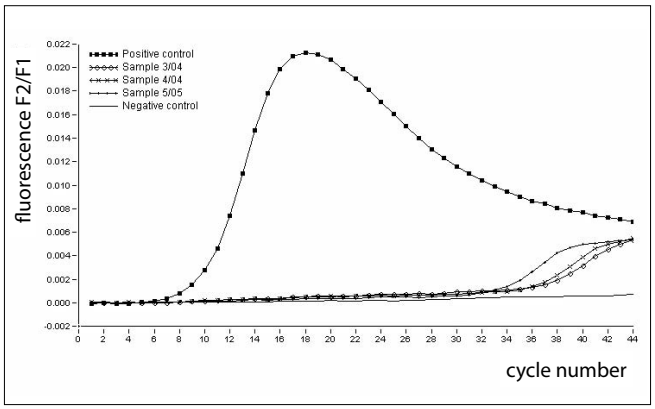

Fig. 2. *Analysis of PCR. With growing number of cycles during amplification, values of fluorescence at some examinated samples grow, what suggests presence of B. pertussis in examinated samples.*

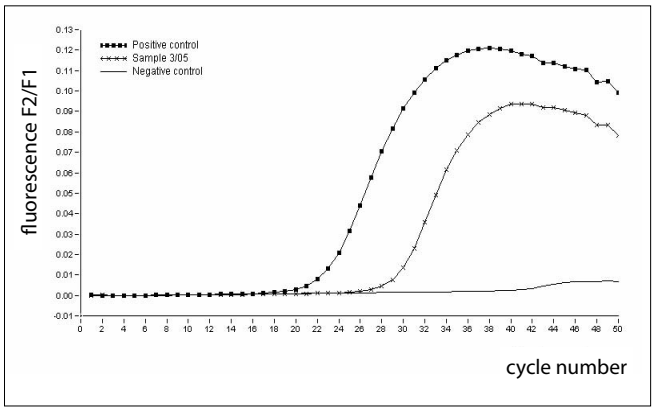

Fig. 3. *Analysis of PCR. With growing number of cycles during amplification, values of fluorescence at examinated sample grow, what suggests presence of B. pertussis in this sample.*

of this sample increased but after the electrophoresis there was no band on agarose gel (Table 2 – sample 26, Fig. 4). After diluting this sample the result was the same. This disputable case was nevertheless considered by doctors as positive because of presence of clinical symptoms of disease.

The presence of *IS481 of Bordetella pertussis* in two control nasopharyngeal swabs from two epidemiologists was not confirmed (Table 3, Fig. 4).

In repeated sample with off-set from little boy, residing in Topoľčany, the result was negative. It is possible that it was because of antibiotics administered to him.

Definitive diagnosis of causative organism in pertussis (whooping cough), *Bordetella pertussis*, is traditionally being made by culture of secretions from the nasopharynx (1). The sensitivity of this test is low, but the specificity is 100%, the culture of BP until recently was the preferred method for diagnosing pertussis even though it takes 4–7 days (1, 15, 16).

Serological tests like KFR or ELISA (detection of antibodies produced against a filamentous hemaglutinin or against pertussis toxin) are more sensitive than culture, but these usually only provide late, or retrospective diagnosis (2).

Also direct antigen detection by a fluorescent antibody test and ELISA techniques have shown problems with specificity (1, 15, 17). There are also two major drawbacks in diagnosing pertussis by serology: it cannot be used in acute phase of the disease, and it can be difficult to differentiate between vaccine effects and a pertussis infection (10).

To overcome these limitations, detection of *B. pertussis* DNA from nasopharyngeal aspirates and swabs has been described using PCR assays, including those targeting the promoter region of the gene encoding pertussis toxin S1 subunit (ptxA) (3, 4), the insertion element *IS481* (5), the adenylate cyclase gene (cyaA) (6) and a region upstream of the outer-membrane porin gene (7). The best material for PCR testing method is a nasopharyngeal swab taking by dacron tampons because previous studies have shown

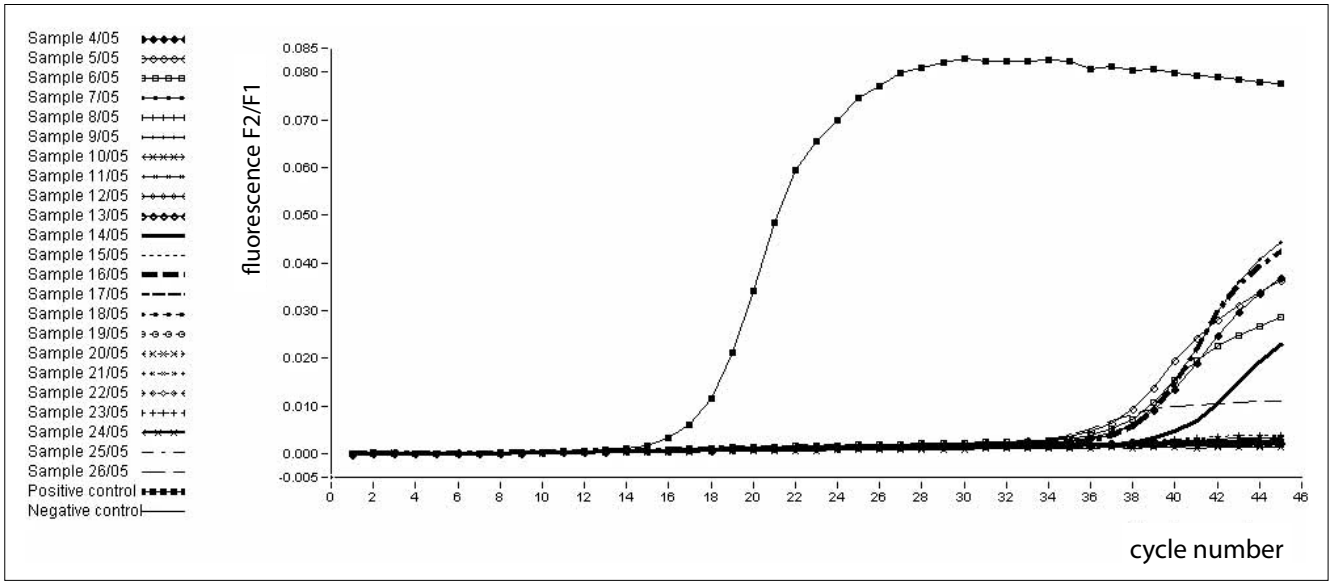

Fig. 4. *Analysis of PCR. With growing number of cycles during amplification, values of fluorescence at examinated sample grow, what suggests presence of B. pertussis in this sample.*

that calcium alginate fiber and aluminium shaft components inhibited PCR reaction (11).

In 2001 Kösters et al. have developed a real-time PCR assay based on the TaqMan technology for detection of *Bordetella pertussis* in clinical samples (9). One year later Reischel et al. have described a real-time PCR technique for detection of *Bordetella pertussis* by using LightCycler instrument and fluorescent labelled probes (10).

Using this method of detection it is also possible to distinguish between *Bordetella pertussis* and *Bordetella parapertussis*. The illness caused by *B. parapertussis* is usually milder than that caused by *B. pertussis*. Simultaneous infection with *B. pertussis* and *B. parapertussis* has been observed in clinical practice.

B. pertussis repetitive insertion sequence IS481 and *B. parapertussis* repetitive insertion sequence *IS1001* were selected as targets for the duplex PCR (10).

Real-time PCR compared to conventional PCR has several advantages: (i) compared to conventional PCR, preparation, pretreatment of sample and processing of reaction are simplified and require far less time (time required for detection is shortened from cca 8 hours to cca 3 hours); (ii) the reaction runs in closed system of glass capillaries, which significantly reduces the possibility of any contamination of analytical sample; (iii) it has a broad spectrum of application, such as the possibility to detect mutations and deletions, analysis of gene expression in the molecular oncology, and others; and (iv) the possibilities to detect pathogens, which are impossible or difficult to cultivate, or they occur in such a small quantity that they cannot be detected by other methods (10, 18). Recent studies (3, 5, 19) suggest that PCR based assays and real-time PCR based assays (9, 10) are more sensitive than culture for detection of *Bordetella pertussis* in nasopharyngeal specimens. Serological methods have shown low sensitivity and specificity too (1, 17). The PCR based assays give us relevant results till few hours, whereas final isolation and identification with culture generally take 3 to 5 days.

Some studies (20, 21, 22, 23) show to significant cross-reactivity of *IS481* based PCR assays with *Bordetella holmesii* genomic DNA. But from the clinical point of view, however, the risk of *B. holmesii* giving false positive results by *B. pertussis IS481* PCR should be minimal because up to now it was found only sporadically in patients with respiratory symptoms (10, 21). *B. holmesii* accounted for less than 4% of *Bordetella spp.* cultured from patients with pertussis-like illness (23, 24).

IS481 based PCR assays could be considered as a valuable addition to pertussis diagnostic testing (10).

Bordetella pertussis is a rare disease in this time so we found these cases clinically and epidemiologically very interesting.

CONCLUSION

In conclusion we consider PCR and real-time PCR based assays to be suitable methods for clinical and epidemiological practice and are also an efficient tool for prevention of epidemic and avoiding negative results due to administered antibiotics.

Moreover, the real-time PCR based assays have some advantages over classical detection methods (cultivation and serology) and conventional PCR based assays, too. It eliminates the need for post-amplification handling, thereby reducing the risk of contamination and also decreases total analysis time (18, 25).

The laboratories equipped with these techniques and possibilities really poses a useful and efficient tool for the prevention and therapy of infection disease.

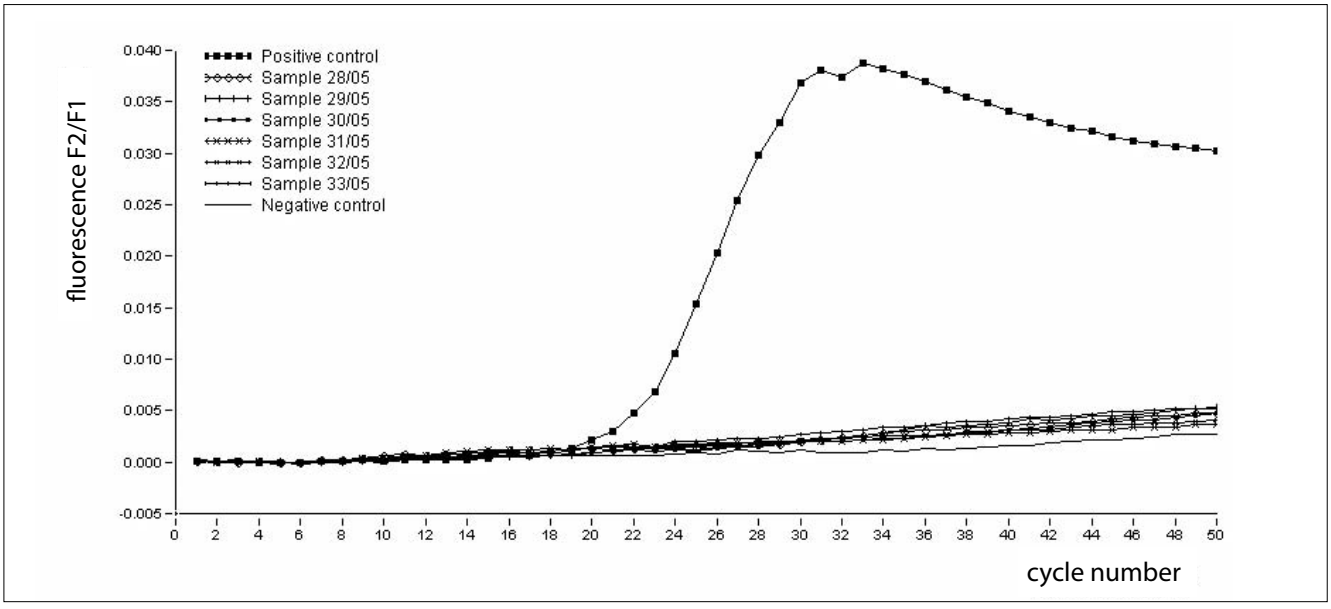

Fig. 5. *Analysis of PCR. With growing number of cycles during amplification, values of fluorescence at the examinated samples did not grow, examinated samples are negative.*

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