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MILÁČEK-VITOVEC STAINING METHOD VERSUS CONVENTIONAL AND MOLECULAR ONES FOR DIAGNOSIS OF *CYCLOSPORA CAYETANENSIS* INFECTION

By

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Abstract

Stool samples from 150 immunocompromised patients (including 120 Gastrointestinal [GI] symptomatic and 30 GI asymptomatic) suspected to have *Cyclospora cayetanensis* infection were examined to evaluate the validity of Miláček-Vitovec staining method in comparison with kinyoun stain for the diagnosis of cyclosporiasis. All results were confirmed with nested PCR as a gold standard. Validation parameters included sensitivity, specificity, accuracy index, positive predictive value and negative predictive value. In addition, infection intensity was compared among all positive samples. DNAs of *Cyclospora* oocysts were detected among 30/120 (25%) of GI symptomatic patients versus 1.7 % of GI asymptomatic (5/30). Miláček-Vitovec stain showed more validity than kinyoun with a sensitivity reached 80% versus 73.4% (28 versus 25 from 35 cases) while the specificities of showed 90.4% (104/115) versus 81.7% (94/115), respectively. Mild infection intensity was detected among 10 patients only three of them (30%) were detected by Miláček-Vitovec stain while only one (10%) was detected by Kinyoun, Moderate infection were identified among 10 patients all of them were diagnosed by Miláček-Vitovec stain and 9 (90%) was detected by Kinyoun stain. Heavy infection was detected among 15 patients all diagnosed with Miláček-Vitovec stain while 14 were detected with kinyoun.

Key words: *Cyclospora cayetanensis,* Miláček-Vitovec staining, Conventional & Molecular ones

Introduction

Intestinal human coccidian parasites *Cyclospora cayetanensis* have emerged as a significant human gastrointestinal pathogen worldwide that causing prolonged, but self-limited diarrhea in immunocompetent patients, while in immunocompromised, diarrhea may be prolonged, severe and fatal (Ortega and Sanchez, 2010). Cyclosporiasis occurs in persons of all ages (Eberhard *et al*, 1997). Generally, the infected patients may experience GIT symptoms such as abdominal cramping, diarrhea (about 6stools/day in acute form), anorexia, and nausea, beside other constitutional symptoms such as flu-like symptoms, fatigue, weight loss, myalgia, bone ache, joint pain and fever. Routine diagnosis of this parasitic infection may be ignored especially in developing countries (Seyrafian *et al,* 2011). The presence, frequency, and distribution of *Cyclospora* parasites in patients with a deficient or suppression of immune system are very important, as it impacts not only the quality of life of these patients but may also have effects on the disease course, therapeutic efficacy and tolerability, and mortality (Seitz and Trammer, 1998). Therefore, improved diagnostic tools for these parasites will feature higher specificity and sensitivity to facilitate more accurate and earlier diagnosis, so that appropriate therapeutic intervention may be initiated in a timely manner (Jiménez-Cardoso *et al*, 2013). Diagnosis of C*yclospora* was done by detection of oocysts in fecal samples using light microscopy by Modified acid-fast (Dixon *et al,* 2005). Due to the extensive variability on the staining of the oocysts as well as interference with particulate matter within the feces the proficiency in identification of *Cyclospora* oocysts by light microscopy has been a challenge (Ortega and Sanchez, 2010). Autofluorescent properties of the oocysts as well as interference with particulate matter within the feces beside the intermittent and low level of oocysts shedding complicate epifluorescence microscopic examination (Chacin-Bonilla, 2010) but

this type of microscopy was not widely available in many laboratories especially in developing countries (Hussein, 2017). Sporulation study is one of the diagnostic tools but it is time consumed and not all of oocysts ablity to sporulate (Ortega and Sanchez, 2010). Also, no immunological or serological diagnosis are available so cyclosporiasis can often be misdiagnosed because of the oocyst detection is the main diagnostic issue (Garcia, 2007). Due to these limitations, molecular-based techniques are more sensitive, specific and time-efficient. Although PCR relies on the genetic information of *C. cayetanensis* yet, did not depend on the subjective nature of the microscopic (Varma *et al*, 2003). But, this method is expensive, and labor-intensive and the need for specialized equipments and experiences (Ortega and Sanchez, 2010). On the other hand, immunodiagnosis using serological assays to determine human exposure to *Cyclospora* were yet available (Garica, 2007). The new staining technique overcomes the low sensitivity of conventional staining method is an urgent issue. Aniline-carbol-methyl violet stain of Miláček-Vitovec was successively used in staining and diagnosis of *Cryptosporidium* oocysts (Miláček and Vitovec, 1985; Holubová *et al*, 2016).

In this study, the validity of Miláček-Vitovec stain as a new diagnostic stain in the diagnosis of cyclosporiasis was compared to kinyoun stain and confirmed by PCR.

Patients and Methods

Patients selection :Fecal samples were collected from 120 from the gastrointestinal tract (GIT) symptomatic and 30 were from GIT asymptomatic patients seeking for medical help and attending gastroenterology clinic and different clinics of Suez Canal University Hospitals, Ismailia. Verbal consents were obtained from the patients and all of the procedures were conducted according to the ethical standard approved by the institutional human ethics committee, Faculty of Medicine, Suez Canal University, Egypt.

Fresh stool samples were examined macroscopically. Samples were examined by light microscopy of wet mounts and then by staining (Garcia, 2007). Fresh stool samples were divided into two parts; the first one was concentrated and sediments were stained for microscopical diagnosis and the second part was preserved in -20ºC for molecular diagnosis as it is (fresh stool) (Ortega *et al*, 1993). Each Stool sample was concentrated with mixing of 15 ml saline (0.9% sodium chloride) to 5 g or 5 ml of feces and filtrated through four layers of gauze to remove the coarse material. The filtrate was centrifuged at 800 x g for 5 min. The supernatant was eliminated and the sediment was examined directly by wet mount. A final volume of 1-2 ml was mixed with 10 ml of Sheather's sugar solution that has a specific gravity of 1.27 and centrifuged at 600 g for 20 min to float many oocysts. The float fluids were collected and examined for the presence of *C. cayetanensis* oocysts after staining by modified Kinyoun (Garica, 2007) and MV (Miláček and Vitovec, 1985).
Miláček-Vitovec staining method: The stain includes two solutions (Miláček and Vitovec, 1985), the first one is aniline-carbol-methyl violet that composed of 0.6gm methyl violet, 1 ml aniline, 1gm phenol, 30ml 96% ethanol and distilled water added to reach 100ml. The solution was lifted for one day and after filtered. The second solution is composed of1%tartrazine in 1 % acetic acid. Dry smears were put in methyl alcohol for 5 minutes at room temperature. The smears were stained with aniline-carbol-methyl violet for 30 minutes and after that rinse in tap water. The smear then differentiated in 1-2% sulphuric acid for 30 seconds tile the slide was pale blue-violet and followed by rinsing with tap water. Finally, the stained smears were put in tartrazine solution to counterstained then rinse briefly with tap water. After the slides became dry the smears were examined.
Intensity of infection: The numbers of oocysts on slides stained by Kinyoun's acid

fast were counted (Mc-Lauchlin *et al*, 1999). The means for 20 fields were calculated at x400 magnification. The numbers of oocysts observed were categorized as follows: None: no oocysts, (+): < 1 oocyst , (++): 1-10 oocysts, and (+++): 11- 100 oocysts. If no oocysts were seen in 20 fields, the entire slide was examined thoroughly before the sample was considered negative (Garcia, 2007).

Molecular diagnosis of *C. cayetanensis*: DNA extraction was obtained from 0.5g stool specimen homogenized in 8ml of distilled water and filtered through a 300 µm-mesh sieve then 3 µl of ether was added, and the water-ether mixture was vortexed for 1 min and centrifuged at 700×g for 2min. The pellet was diluted in 100 µl of water after decanting the supernatant then DNA was extracted using a QIAmp tissue kit (Qiagen, Hilden, Germany). The extracted DNA was stored at -20°C until further use. Controls DNA stool samples positive and negative for *C. cayetanensis* oocysts were obtained from our previous study (Hussein *et al*, 2016).

PCR was performed using 18S rRNA as the target in a nested PCR assay using gene oligonucleotide primers F1E (5'-TACCCAAT GAAAACAGTT-3') as the forward primer & R2B (5'-CAGGAGAAGCCAAGGTAG-3') as the reverse primer for the external round of PCR to amplify a primary amplicon of 636bp. Then inner primer pair F3E (5'-CCTTCCGCGCTTCGCTGCGT-3') & R4B (5'-CGTCTTCAAACCCCCTACTG-3') as the forward and reverse primers, respectively were used to generate generated a 294bp amplicon. PCR was conducted at two amplifications rounds (primary and secondary) according to Orlandi et al. (2003). In a total volume of 50 µl, the primary amplifications round was performed. It contained 20 µl of template DNA, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 Triton X-100, 2 mM MgCl2, 200µM of each dNTP, dCTP dGTP and dTTP, 0.2 µM of each primers F1E and R2B and 1.25 units of Taq DNA. An initial activation of the HotStarTaq DNA polymerase at 95^{o}C for 15 min was begun. Then, the denaturation cycle was performed at 94oC for 30 seconds for 35 cycles followed by annealing at 53oC for 30 seconds and primer extensions at 72^{o}C for 90 seconds. Then a final extension cycle followed at 72oC for 10 min in an ABI 2720 thermocycler (ABI Biosystems, USA). After that, the second round was conducted in a reaction volume of 50 µl and 5 µl of the primary round product was used as a template. Reaction component concentrations were the same as in the primary round with the following exceptions: the primers used were F3E and R4B and the annealing temperature was increased to 60oC. The nested PCR products were examined after electrophoresis on a 1.5% agarose gel, stained with 0.2µg/ ml of ethidium bromide and visualized on a UV transilluminator.

Statistical analysis: Validation parameters included sensitivity, specificity, accuracy index (positive predictive value and negative predictive value. Significance difference was measured by chi-square test or Fisher exact test. P-value ≤ 0.05 was statistically significant.

Results

In the present study, 35 Cyclospora-positive fecal samples were confirmed with nested PCR by the production of *Cyclospora*-amplicons out of 150 detected by either of Miláček-Vitovec or kinyoun stain. Variably kinyoun stain oocysts measure 8-10µm in diameter (Fig.1a,b,c) as the sporulated and unsporulated oocyst have variability on staining reaches to completely unstained oocyst. By Miláček-Vitovec stain all the sporulated and unsporulated were stained. On the other hand, sporulated oocysts were presented darker than unsporulated (Fig 2 a,b). DNAs of *Cyclospora* oocysts were detected among 30/120 (25%) of GI symptomatic patients versus 1.7% of GI asymptomatic (5/30) (Fig. 3a &b). Miláček-Vitovec stained only 28 out 35 of true positive so its sensitivity was 80% while its specificity was 90.4% (104/115) with an accuracy index reached 88%. Positive and negative predic-

tive value was 89.8% & 93.7%, respectively. Regarding kinyoun, sensitivity was 73.4% (25/35) specificity was 81.7% (94/115) and accuracy index was 79.1%. Positive and negative predictive values were 76.4 % and 90.1%, respectively (Tabs.1 & 2). As regarding to the intensity of infection, 10 samples had mild infection three of them were detected by MV stain while only one was detected by Kinyoiun, Moderate infection were identified among 10 patients all of them were diagnosed by Miláček-Vitovec stain while 9 was detected by Kinyoiun stain and one was completely missed because it failed to stain. Patients with heavy infection were 15 patients all diagnosed with Miláček-Vitovec stain while 14 detected with kinyoun and missed one was unstained (Tab. 3), with significant differences.

Table 1: Comparison between of MV and Kinyoun stains versus nested PCR as gold standard diagnosis.

		Nested PCR (Gold standard)					
		Positive		Negative		Total	
		No	%	No	%	No	%
MV	Positive	28	80	11	9.6	39	26
	Negative	7	20	104	90.4	111	74
	Total	35	100	115	100	150	100
Kinyoun	Positive	25	73.4	21	18.3	45	30
	Negative	10	28.6	94	81.7	105	70
	Total	35	100	115	001	150	100

Table 2: Evaluations of validation parameters for of MV & Kinyoun stains vs. nested PCR a gold standard.

	Sensitivity	Specificity	Accuracy index	Positive Predictive value	Negative Predictive value
MV	80	90.4	88	89.7	93.7
Kinyoun	73.4	81.7	79.4	76.1	90.4

Table 3 Intensities of infection with each stain

		Mild positive		Moderate positive		Heavy positive	
		No	%	No	%	No	%
MV	Positive	3	30	10	100	15	100
	Negative	7	70	0	0	0	0
	Total	10	100	10	100	15	100
Kinyoun	Positive	1	10	9	90	14	93.4
	Negative	9	90	1*	10	1*	6.6
	Total	10	100	10	100	15	100

*oocyst failed to stain completely, with significant difference

Discussion

Until now, no animal models, tissue culture or monoclonal antibodies are available to study human *Cyclospora i*nfection (Eberhard *et al,* 2000; Karanis *et al,* 2007). Although the acid-fast staining method is considered the easiest and most practical and provides a stained slide it can lead to misdiagnosis due to the variability in staining and confusion with artifacts (Eberhard *et al,* 1997). Lainson (2005) recommended the molecular-based technique as reliable method for *C. cayetanensis* identification.

This study showed that Miláček-Vitovec stain is more valid than kinyoun with no variability on the stain. Chu *et al.* (2004) and Khalifa *et al.* (2004) emphasized that the nested PCR had 100% sensitivity and specificity, therefore, it is the confirmatory diagnostic method, in the present study, for the detecting cyclosporiasis infection. Furthermore, the possibility of cross–detection between the presence of *Cyclospora* and *Eimeria* species by nested PCR is not possible, because no *Eimeria* infection was present yet in human (Pieniazek *et al,* 1996; Relman *et al,* 1996). Although, nested PCR is still the most popular and confirmatory method used to detect *Cyclospora* DNA (Betancourt *et al,* 2002; Steele *et al,* 2003; Jiménez-Cardoso *et al,* 2013; Orozco-Mosqueda et al. 2014; Hussein, 2017). Yadav et al. (2015) proved that PCR assay amplified *C. cayetanensis* DNA in only 89% (17/19) isolates. This variability on the sensitivity of PCR was explained by Orlandi *et al*. (2002) who

stated that the molecular diagnostic methods generally still depend on the ability to isolate and concentrate the parasites from samples matrix as well as ability to prepare a suitable DNA template free of matrix-derived substances that may interfere with PCR results. Also, lacking PCR in many laboratories, particularly in developing countries led to limitations as use in routine diagnosis.

In the present study, 35 *Cyclospora*-positive fecal samples were confirmed with nested PCR out of 150 stained by Miláček-Vitovec and/or kinyoun. The sensitivity of kinyoun stain was very low (73.4% (2535/) and specificity was low (81.7%). Sensitivity and sp-ecificity of Miláček-Vitovec stain in *Cyclospora* oocysts were 80% & 90.4% respectively. There were false results by using the staining techniques compared to PCR. However, the false negative and false positive results were shown by using Kinyoun technique, statistically- higher than that shown by Miláček-Vitovec staining. These data agreed with Betancourt *et al.* (2002), Steele *et al*. (2003) and Khalifa *et al.* (2004) who found that the sensitivity and specificity of staining methods were lower if compared to molecular techniques. Moreover, the modified kinyoun stain showed great variability in the staining pattern of *Cyclospora* oocysts leading to decrease in its sensitivity (Visvesvara *et al*, 1997; Negm, 1998). Also, it was neither timely nor specific (Orlandi *et al,* 2003). All these problems led to limitation of the kinyoun staining diagnostic method (Garcia, 2007). In the present study, Miláček-Vitovec stain was used for the first time in diagnosis *Cyclospora* infection. This present data agreed with Kváč *et al.* (2003) who compared different methods for routine diagnostics of *Cryptosporidium spp* to Miláček-Vitovec stain and found that specificity of both stainings was 95-100%, respectively. Ziehl-Neelsen was more suitable for identification of *C. andersoni* and modified MV for *C. parvum* identification.

As regarding to the intensity of infection, 10 samples had mild infection three (30%) of them detected by Miláček-Vitovec stain while only one (10%) was detected by Kinyoun, Moderate infection was identified among 10 patients all of them were diagnosed by Miláček-Vitovec stain while 9 was detected by Kinyoun stain and one was completely missed because it failed to stain. Heavy infection was detected among 15 patients all of them were diagnosed with Miláček-Vitovec stain while 14 were detected with kinyoun and rest one was completely missed because it failed to stain. The differences were statistically significant.

In case of infection intensity, the microscopic counts of *Cyclospora* oocysts are still the gold standard (Ortega *et al*, 1998; Varma *et al,* 2003). Also, detection limits of nested PCR was 10 oocysts per inoculated fecal sample (Sturbaum *et al,* 1998), no samples were found to be positive by staining and negative with PCR as repeated PCR for all samples positive with staining twice. Sometime, no samples were positive by both stains and negative with PCR.

The infection rate of C. *cayetanensis* was in a hospital in Honduras (2002-2011) with 1·3% (125/9984), based on Ziehl–Nielsen Carbol fuchsin staining (Kaminsky *et al,* 2016). But, a lower infection rate (0·67%, 60/8877) was detected in a pediatric hospital (patients aged<15 years) in Mexico (in 2000–2009), based on morphometric characteristics and PCR (Orozco-Mosqueda *et al,* 2014). A higher prevalence (3·94%, 20/507) was detected in school children (aged 3-14years) in Nepal, with modified acid-fast staining (Bhandari *et al*, 2015), and a markedly higher rate was reported in humans in Italy (27·5%, 11/40), based on quantitative PCR (Giangaspero *et al,* 2015). On the study of Vijay Prakash Singh *et al.* (2015) the prevalence of *Cyclospora* parasites among 62 immunocompromised patients with diarrhea was found to be 4.8% and in immunocompromised patients without diarrhea, no infection was seen. The prevalence of cyclosporiasis was carried out by Ziehl Neelsen staining and PCR among immunosuppressed pa-

tients, including 96 individuals with the human immunodeficiency virus or 77 having acute lymphoblastic leukemia with or without diarrhea. It was 7.3% among diarrheic patients, whereas, the prevalence was zero for *Cyclospora* spp. among non-diarrheic (Jiménez-Cardoso *et al*, 2013).

Conclusion

A Miláček-Vitovec staining for *C. cayetanensis* oocysts proved to be sensitive, specific, accurate and efficient. Thus, can be applied for laboratory diagnosis even in mild and moderate infection in spite of being time consuming than Kinyoun. Molecular methods are clearly more promising than microscopy, however, staining examination is not dispensable as lacking PCR in many laboratories that lead to limitations of its user.

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Explanation of figurres

Fig.1: *Cyclospora* oocyst under light microscopy X1000: Fig 1a Completely stained unsporulated oocyst by Kinyoun stain green; Fig 1b: Partialy stained sporulated oocyst by Kinyoun stain Fig1c:Oocyst unstained by Kinyoun and stained with malachite green.

Fig. 2 Cyclospora oocyst under light microscopy X1000; fig.2a Unsporilated *Cyclospora* oocyst stained with Miláček-Vitovec. Fig 2b. sporulated *Cyclospora* oocyst stained with Miláček-Vitovec

Fig.3 a&b-: Examples of DNA assays of fecal samples by Nested PCR. *M* =a ladder DNA at 100 bp. a; Positive and negative controls in lane 1 & 2 respectively. *Cyclospora* positive samples were present in lanes 4, 5&7 found at 294 bp while lanes 2,3 &6 were negative. f ig 3b; lanes 10,11,12,14,15 positives, lanes 13 &16negatives.

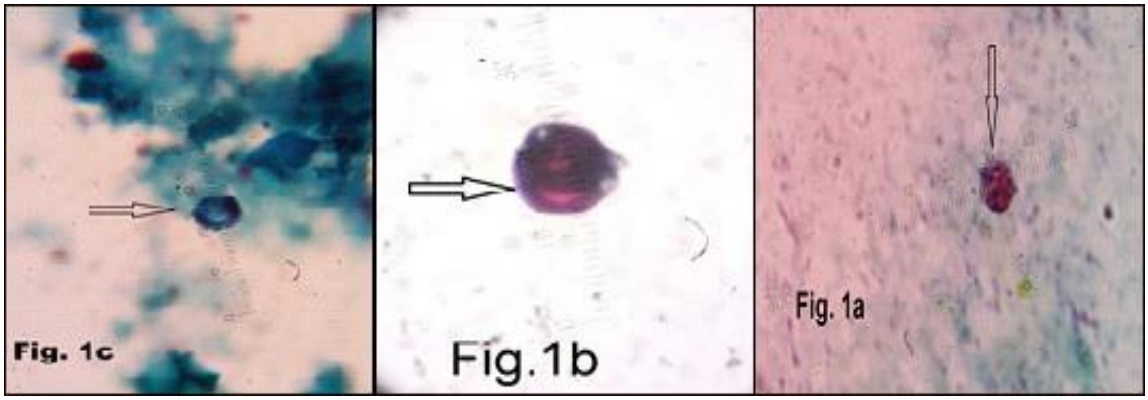


Fig. 1c
Fig.1b
Fig. 1a

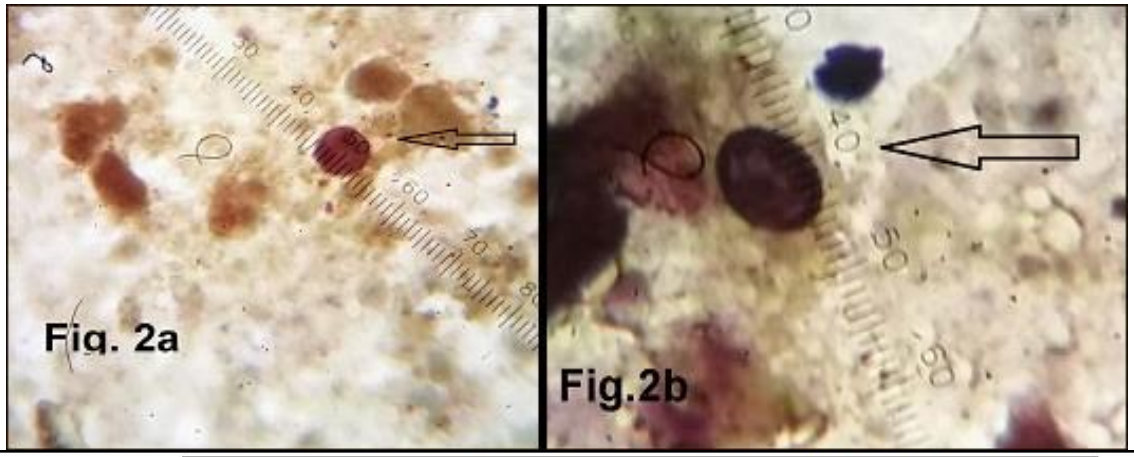
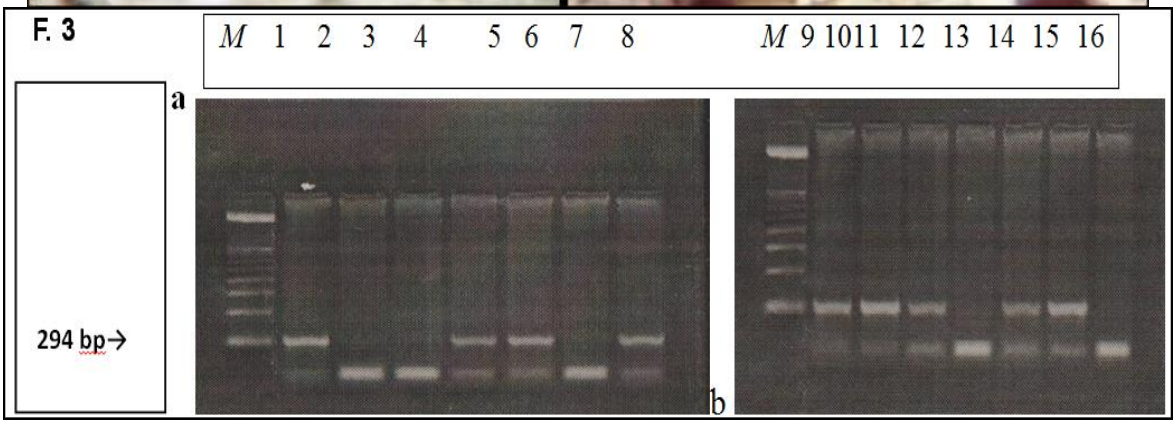


Fig. 2a
Fig.2b

F. 3


M 1 2 3 4 5 6 7 8
M 9 1011 12 13 14 15 16
a
294 bp→
b