

## Adaptation of Microstix®-Candida Slide-test for Diagnosis of Bovine Mastitis Due to Anascogenic Yeasts

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### Abstract

Although a large diversity of mastitis-causing bacteria (150 species, subspecies or biovars) have been documented, an adequate and simple mycological screening for yeast-related mastitis has not been developed. Since yeast incubation normally lasts longer than the majority of bacteriological ones, usually a treatment is administered before the results of microbial cultures are obtained. Therefore, a simple test has been needed for detection of yeast-related mastitis, the results of which can be read in parallel to bacteriological screening.

Application of Microstix®-Candida assay was first checked for its specificity in standard *Candida albicans* (Oxford strain) cultures (phase I) and then in the other 36 strains of yeasts isolated from clinical mastitis cases during field investigations (second phase). Next, a test was implemented for identification of pathogens in 9 cohorts (1,200 cows) from red-white Polish-Friesian breed in which the dynamics of mastitis were traced by bacteriological and mycological assays along year-long observations.

The data obtained showed that apart from frequent infections (~50-70%) proved by using pure microbial cultures, also mixed (bacterial-fungal) infections (0-25%) appeared as well as negative sieves (in the range of 0-45%). Mycological assays revealed a significant fraction of mixed infections during both lactation and the dry period. Strepto-staphylococcal infection was thus identified, however, its proportion strongly decreased after the introduction of antibiotics (after sensitivity to microbial agents was determined). Microbial monitoring was improved in these herds by parallel application of bacteriological and mycological (particularly Microstix®-Candida) tests.

Microstix®-assay appears to be useful for mycological diagnosis in field conditions of infected (mastitis) herds. Its advantages include easy performance and short incubation time that is only 24 h at 37 °C (72-96 h at room temperature). This allowed demonstrating the cow's udder yeast infection simultaneously with the first reading of bacterial cultures. Its specificity was confirmed against standard *Candida* Oxford strain, and sensitivity (100%) was confirmed in mycological cultures (36 strains) and biochemical assays.

*Bovine mastitis, mycotic mastitis, bacteriological mastitis, cow mastitis diagnosis, Candida assay*

The presence of bovine mastitis in herds is a natural consequence of interaction among many constitutional and environmental factors taking place within the cow's mammary gland. Mastitis control and prevention requires an effort in improving the milking system, cattle feeding and hygiene, housing conditions and breeding policy. Currently in Poland, there is no public or centrally managed mastitis control scheme but the economic advantage of improving cow's udder health is emphasized by dairy farmers, which often motivates them to take their own uncoordinated measures. An epidemiological study by Malinowski (2001) indicated that nearly 50% of milking cows in Poland might have been affected by a subclinical (SCM) or clinical (CM) mastitis, sometimes causing extreme economic losses in cattle farming. Our current knowledge on the aetiology and pathway of this disease marks contagious cases and constitutional non-infectious factors. Primarily, only bacterial infections were considered, and very rarely viral or mycotic ones were recognized.

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Therefore, the majority of mastitis control schemes (MCS) recommended intra-mammary application of antibiotics during both lactation and mammary gland involution as well as application of disinfectant solutions before and after milking.

Bacteriological and mycological diagnosis of specimens from 2,374 cows' quarters within SCM was performed by Klimaitė (2005) and revealed that pure cultures of bacteria or yeasts indicated only 26.4% cases of bovine mastitis, while up to 64% were caused by mixed infections (bacterial-fungal). Moreover, in approximately 9.4% of SCM cases no bacterial or yeast strains were isolated (negative sieves). Among the mixed infections, most often yeast-like fungi with staphylococcal (coagulase-positive or negative) strains co-existed.

Great diversity of mastitis-causing factors encompasses about 150 species of bacteria; however, adequate mycological diagnosis (yeast-related mastitis or mixed bacterial-fungi infections) should also be required. Usually animal health and welfare dictated a treatment of CM before the diagnostic microbial cultures and antibiotic sensitivity assays would appear. After the intra-mammary use of antibiotics the health of the cow's udder is often affected by yeast-related mastitis or mixed bacterial-fungal infections that deteriorate the process.

Microstix®-Candida slide-test was prepared by the Ames-Company (USA) for laboratory diagnostics of the vulvovaginitis mycetica in women. Its adaptation for diagnostic purposes for bovine CM was due to the importance of anascogenic yeasts screening in field conditions. Since incubation time of this test is very short, the results may be obtained simultaneously with bacteriological reading.

### Materials and Methods

#### Microstix®-Candida assay

Microstix®-Candida kit packet contains 25 reagent strips with micro-bases proposed by Nickerson for cultivation of yeasts and 25 folio sacks in which the inoculated strips can be incubated for 24 h in a wet chamber at 37 °C or for 72-96 h at room temperature. Its potential application was experimentally checked in standard cultures (first phase) and introduced to the diagnosis of bovine CM in field conditions (second phase) where both pure yeast-related mastitis cases or mixed bacterial-fungal infections can be present. The standard strain of *Candida albicans* (ATCC, No 10231) was used as laboratory reference, while inoculum at initial density of  $1.4 \times 10^{13}$  cfu/ml was produced and its decimal dilutions on sterile milk samples were prepared.

#### Experimental cases

Objects of the field trial (second phase) consisted of 9 cohorts (1,200 individuals) of highly yielding cows (> 7,000 kg of milk/year) from a Polish-Friesian red-white breed that fed on pasture and were milked at the stalls to pipeline installations (de Laval). Among the cows from these 9 cohorts, germ populations present in lactation or during udder involution were compared. Both bacteriological and mycological sowings of milk samples were performed, and then the bacteriological diagnostics was modified for mycological screening. The diagnostic base of yeast-related mastitis by both standard mycology and Microstix®-Candida kit packet was established. Yeast-like strains were identified based on classical methods or using commercial API tests (API 20 C and API 20 C AUX).

#### Standard mycology

According to methods currently available in mycology (Elad et al. 1995; Gonzalez et al. 2001; Gedek 1968; Klein 1981; Lodder 1970; Richard et al. 1980) all the strains of yeast-like fungi were plated. The morphology of these yeast macro-cultures (colony characteristics) grown on blood agar (containing 5% lamb blood enriched by addition of 0.1% aesculin) as well as on the surface of solid-base proposed by Sabouraud with detreomycin (0.5 mg/ml) or Pagano-Levin solid-base was then compared. Simultaneously, microbial smears with Gram and/or Giemsa staining were prepared and parallel micro-cultures microscopically analyzed. Microscopic glasses were covered with a thin layer of Difco agar, then sieved, and incubated in a wet chamber at 37 °C for 5 days. Micro-cultures of the tested strains were microscopically analyzed on the 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> day of culture, and photographically documented.

#### Biochemical assays

Yeast ability for fermentation was evaluated in liquid medium containing the following carbohydrates: glucose, galactose, saccharose, lactose, maltose or raffinose (zymogrames) as proposed by Lodder (1970). To each test-tube 0.1 ml samples of the tested yeast strain were instilled and incubated at 37 °C for 14 days. The ability to

generate acid (dye) or gas (Durham tubes) during this process was assessed after 24, 48 and 72 h, as well as after 7 and 14 days. Assimilation of carbohydrates or nitrogen compounds and ethanol by these samples (auxonograms) according to Vanbreuseghem (1966) or Barnett and Pankhurst (1974) was assessed. Every culture was performed at 37 °C and kept for the next 3 days while the microbial growth was macroscopically evaluated. Only in auxonograms with ethanol, the typical rings on the test-tube wall and the turbidity of its content (growth of yeasts) were recorded. The ability of tested strains to urease production was checked on Christensen's solid base modified by Philpot (1996). The cultures were sieved on the base containing urea as well as on the control base without urea and incubated at 37 °C for 5 days. As a positive result, the light-yellowish dye changed into the red-violet.

#### Statistical analysis

Statistical classification of mastitis cases was performed using Gauss test. Significant differences were assessed at  $p < 0.01$ ,  $p < 0.05$  and  $p < 0.001$ . Test of variance analysis was performed, and the means were considered significantly different when verified by Student's *t*-test (Szwabe 1987). For interdependence between the qualities of variants  $\chi^2$  test was applied.

### Results

Comparison of germ populations causing CM in lactation (Fig. 1a, see page 116) and at dry udders (Fig. 1b, page 117), and after 2, 4, 6 and 8 months from the onset of antibiotic-therapy implemented to the herds infected with *Staphylococcus aureus* is shown in Fig. 1. Results of pathogen identification included exclusively bacteriological sieves (upper panel) and its microbiological modification after analysis of mycological cultures (lower panel). The results showed that beside frequent infections (~50-70%) by pure microbial cultures, also mixed infections of the udders (0-25%) as well as negative-sieves (0-45%) appeared. In the cohorts studied here, the increase of this negative-sieves proportion (fraction) turned out to be significant after implementation of antibacterial therapy both for CM in lactation and in the dry period (taking into account evaluation of the alveolar = vesicular raw milk). While the increase in negative-sieves after intra-mammary therapy during involution may be called a success, a more precise analysis of CM in lactation is needed. The noted increase in sterile fractions after 2, 4 and 6 months was found to be highly significant (Fig. 1ab), however, before the end of the 8<sup>th</sup> month this phenomenon was not further observed. In lactation (Fig. 1a), a marked increase of CM fraction caused by *S. aureus* after 8 months was considered highly significant ( $p < 0.001$ ). Contrary to this observation, a significant decrease in the mixed infections was noted due to the presence of environmental streptococci. The observed secretions from dry udder quarters (Fig. 1b) compared to initial tests showed a significant increase ( $p < 0.001$ ) of new intra-mammary infections (IMI) in which the CNS occurred after 8 months (Fig. 1b).

#### Yeast-related mastitis

Single cases of yeast-related mastitis were detected (Fig. 1a; lower panel), but since they were not observed at the starting point, their increase was highly significant ( $p < 0.001$ ). The mycological tests revealed significant presence of bacterial-fungal mixed infections detected both at the quarters in lactation and in udder involution. Indeed, bacterial screening (upper panel) was reconfirmed, showing strepto-staphylococcal infections, but they were less frequent than the previous ones, and their proportion, due to application of antibiotics in lactation, was significantly decreased. After mycological reading, the number of such cases was reduced, but concomitantly the co-existence of 3 germ species at the same inflammatory site (2 species of bacteria and 1 fungus) was revealed. In the same way, a significant decrease of staphylococcal infections was noted (*S. aureus* and CNS),  $p < 0.001$ .

#### Macro- and micro-culture morphology of yeast-like fungi

Colony morphologic characteristics as well as microscopic data for particular yeast strains in the field trial after previously performed cultures from CM cases had been analyzed. The colonies grown on Sabouraud's solid-base were macroscopically visible after 48-72

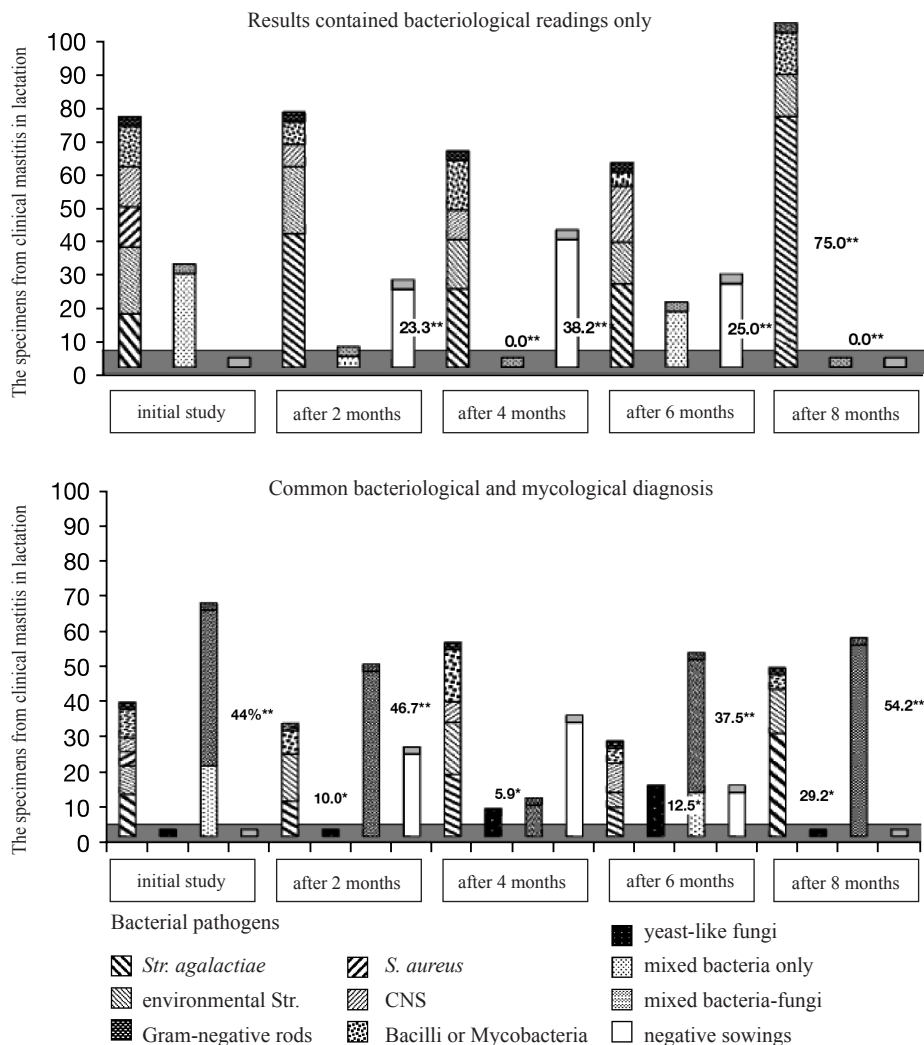
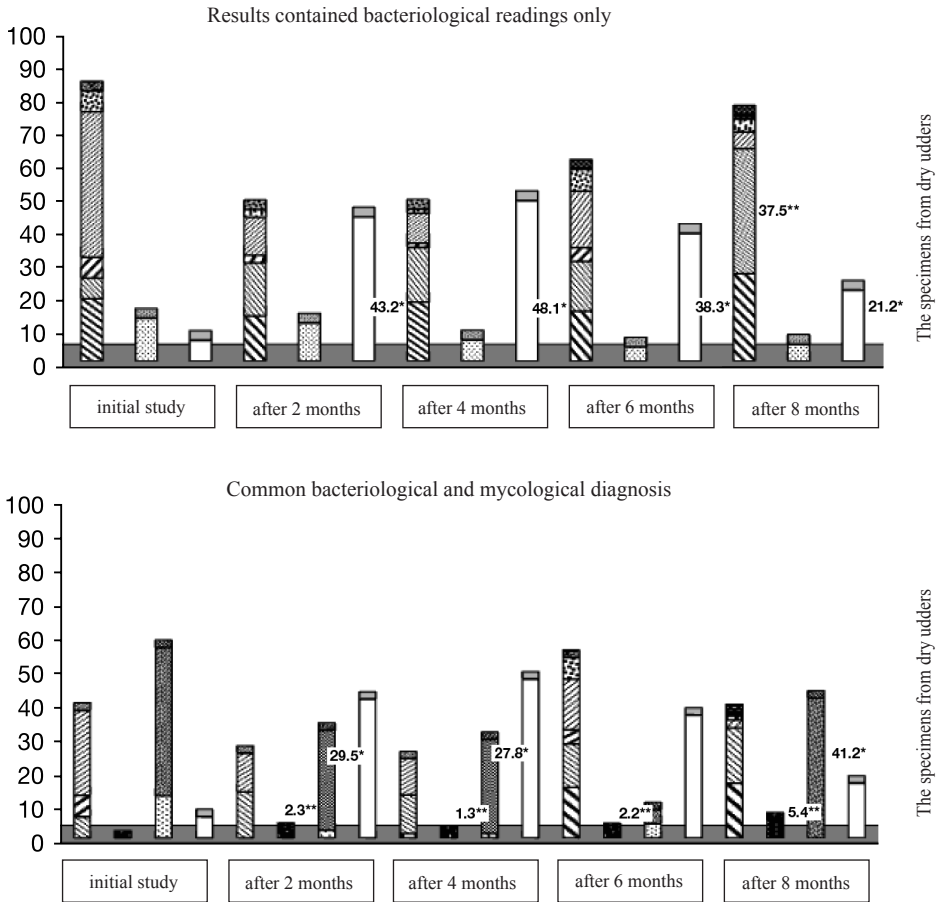


Fig. 1a. Modification of bacteriological mastitis diagnosis caused by the introduction of mycological tests (lactating cows)

h with the optimal diameter appearing no earlier than after 5 days. The reading after 14 days of incubation at humidified chamber occasionally improved its detection (< 10% of cases). The outline visible on Pagano-Levin base after 48 h of culture achieved an optimal diameter of 3 mm between 3 and 5 days of incubation.

Among the typical mycological bases (pH 5.5), a growth of yeasts on blood agar (pH 7.4) was slower and its detection ability was worse. Although at the initial phase the growth had buttery consistence and typical smell (berry or food yeast), the colonies were then differentiated. After 24-48 h of incubation they resembled pin pricks (similarly to the initial growth of some streptococci), after 48-72 h the white and round colonies (similar to *S. epidermidis*) were transformed but optimal diameter (~3mm) was not visible until 3 to 5 days.

When the cultures were analyzed microscopically, usual morphological elements such



Abbreviations:

CNS - coagulase-negative species from *Staphylococcus* genus

\*  $p < 0.05$  Str. - species from *Streptococcus*

\*\*  $p < 0.001$  S. - species from *Staphylococcus* genus

Fig. 1b. Modification of bacteriological mastitis diagnosis caused by the introduction of mycological tests (dry cows)

as blastospores, arthrospores, pseudomycelium or mycelium were revealed (Plate VIII, Fig. 2). All the strains of yeasts studied revealed negative results at the filamentation-test, contrary to positive results obtained with the standard strain. Therefore, it could be confirmed that none of the identified strains was classified as *Candida albicans*.

Considering the repeated biochemical assays and the ability of fermentation and assimilation of carbohydrates (or other chemical substances), taxonomy of the 2 tested strains was impossible. The highest ability of assimilation of nitrogen (from  $(\text{NH}_4)_2\text{SO}_4$ ) was shown for the strains: *Candida guilliermondii*, *C. lipolytica*, *C. solani*, *Trichosporon cutaneum*, *Pichia membranaefaciens* and *Rhodotorula minuta*. The strains of *C. krusei* assimilated nitrogen slowly. Only strains of the *C. solani* showed the ability to assimilate nitrogen from  $\text{KNO}_3$ . The other species of tested yeasts revealed negative results of

nitrogen assimilation. *Candida guilliermondi*, *C. kefir*, *C. curvata*, *C. solani*, *Trichosporon cutaneum*, *Pichia membranaefaciens* and *Rhodotorula minuta* assimilated ethanol as a source of carbon. Thus, it could be confirmed that cases of yeast-related mastitis in the region of Northern Great Poland were most frequently caused by *Candida kefir* (9 strains). The *C. krusei* (5 strains), *C. curvata* (4), *C. brumptii* (4), *C. guilliermondi* (4) and *Trichosporon cutaneum* (4) were rarely isolated. Moreover, strains of *C. lipolytica*, *C. solani*, *Pichia membranaefaciens* as well as single cases of *Rhodotorula minuta* were also shown. The data presented here indicated 36 strains isolated in pure cultures from the cows that were affected by yeast-related mastitis.

Examples of these yeast species in field conditions were thus obtained. Together with the standard strain of *Candida albicans* (ATCC 10231) they were used for the Microstix®-Candida slide-test to prove its diagnostic value for detection of yeast-related mastitis or mixed bacterial-fungal infections of the cow's mammary gland. Both positive and negative results of milk sample sowings from the kit packets are found in Plate IX, Fig. 3. Positive reactions (intensive brown staining of the micro-base) with strains of *C. guilliermondi*, *C. brumptii*, *C. curvata*, *C. solani*, *C. lipolytica* and *Pichia membranaefaciens* were observed. For all the remaining species of anascogenic yeasts that were experimentally employed as well as for all the positive samples which were studied in field conditions, a typical brown staining of the strip was visible, however, with different colour intensity. Among the strains of *C. krusei*, *C. lipolytica* and *Pichia membranaefaciens*, the most intensive staining was observed. In the other tested species a less intensive stain of micro-bases was provoked, although density of these yeast inocula was affected. Independently of colour intensity, they were easily distinguishable from the control samples (aseptically collected from healthy cows).

## Discussion

The manufacturer of the Microstix®-Candida slide-test studied here (that was primarily invented for laboratory diagnosis of vulvovaginitis) recommended it for *C. albicans* detection. According to the enclosed instructions, it may be also applied for detection of other infections of the genital-urinary tract due to *C. krusei*, *C. kefir*, *Rhodotorula rubra*, *Trichosporon cutaneum*, *Cryptococcus neoformans* and *Geotrichum* sp. The study confirmed not only its general use for veterinary practice but specifically for detection of yeasts isolated from milk samples. A general advantage is its simplicity and easy performance as well as short incubation, which at 37 °C lasted only for 24 h. Moreover, it resembled classical mycological culture sensitivity (> 95%), since brown staining of the positive micro-bases was easy to distinguish from negative controls (white colour), even if the density of yeast-inoculum was lower than 10<sup>3</sup> cfu/ml. In addition, shades of positive brown staining indicated its specificity to detect different yeast species, although visually this effect was difficult to measure objectively. Therefore, the data presented may suggest that this test can be recommended to diagnose yeast-related mastitis and mixed bacterial-fungal infections of the cow's mammary gland. Its positive reactions with *C. guilliermondi*, *C. brumptii*, *C. curvata*, *C. solani*, *C. lipolytica* and *Pichia membranaefaciens* (intensive brown staining of micro-base) were observed. For all the remaining strains of anascogenic yeasts a typical brown staining of the strip was visible, however, with less colour intensity. The most intensive staining was observed with the strains of *C. krusei*, *C. lipolytica* and *Pichia membranaefaciens*.

The study presented here can be summarized to the advantage of Microstix®-Candida slide test, particularly for its simple and easy performance as well as short incubation required in which at 37 °C only 24 h can be sufficient. This period of time can be even shortened as for isolation of yeast-like fungi during classical mycological cultures. In conclusion, Microstix®-Candida slide test may be useful for routine diagnosis of yeast-related mastitis

or mixed bacterial-fungal infections of the bovine mammary gland, especially in field conditions. It allows showing the etiology of the cows' udder infection caused by yeast-like fungi simultaneously to the first reading of bacterial cultures. Although the proof presented here is encouraging, we may still need to search for other means of simple and quick pathogenic yeast identification.

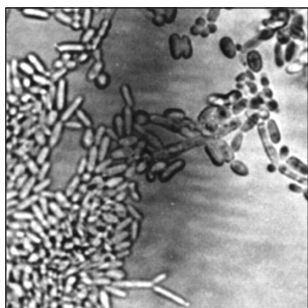
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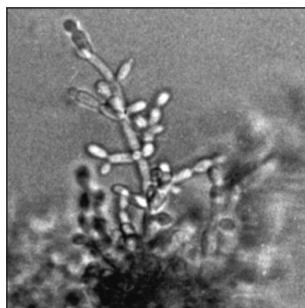




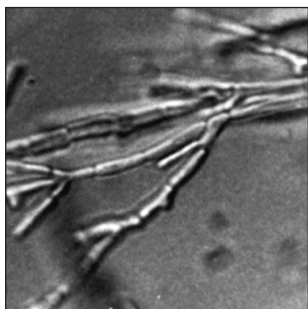
Fig. 2. Morphological elements observed in the micro-cultures of tested yeast strains  
*Candida kefyr* *Candida krusei*



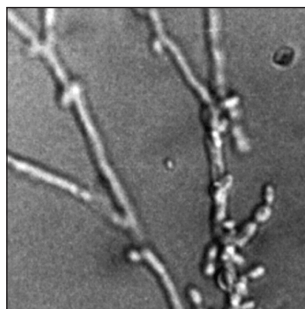
A blastospores at the  
moment of budding



B tree-like arrangement  
of the blastospores

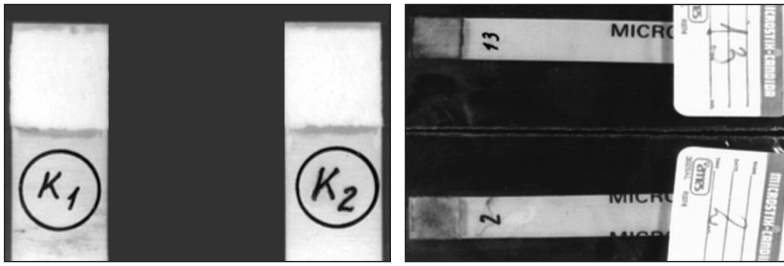


C pseudomycelium  
ramified



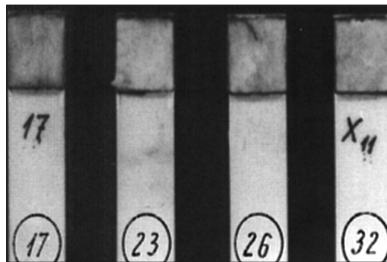
D ramification of the mycelium  
as arthro- and blastospores

Fig. 3. Sowings at the micro-bases of the Microstix® Candida slide-test



a negative results - the presence  
of yeasts not detected

b the positive results  
reagent-strips on the folio-sacs



c the positive results  
presence of yeasts is confirmed

Explanation:

K<sub>1</sub> - a sterile sample of regenerated milk is made from powder  
K<sub>2</sub> - a raw milk sample, collected aseptically