Selection of nutritive compounds to improve growth and indicative properties of the DTM-Expert medium for dermatophytic fungi

V A Savinov, R S Ovchinnikov, A G Gaynullina, A V Khabarova and A V Kapustin
Federal State Budget Scientific Institution “Federal Scientific Centre VIEV” (FSC VIEV). Ryazanskiy prospect, 24, 1, Moscow 109428, Russia
E-mail: visik06@mail.ru

Abstract. The study describes optimization stages of Russian medium “DTM-Expert” for express detection of dermatophytic fungi. In order to improve the growth promotion and indicative properties of the medium, several variations of nutritive compounds were studied. The medium containing mannitol and glucose as carbon sources showed the best indicative and growth promotion properties. The pH optimum for dermatophytes growth on the DTM-Expert was in the range 4.7-5.5. The indicative properties of the medium were optimal at an initial pH value of 4.8. No significant differences were found when using bromothymol blue as an indicator compared to phenol red. The diagnostic efficiency of the optimized version of the DTM-Expert was 100% while the percentage of false positive results was 5.6%. The efficiency of the control medium was 87% accompanied by 9.2% of false positive reactions. The average reddening time was 10 days on the DTM-Expert and 11 days on the control medium.

1. Introduction
Various methods are used to diagnose dermatophytosis in animals, among which inoculation on DTM-type medium (DTM – dermatophyte test medium) is gaining popularity. Initially, the medium was developed for medical purposes [1], but after a while inoculation on DTM became widely used among veterinary laboratories for diagnosis of animal dermatophytosis [2, 3, 4, 5, 6, 7]. The principle of using the medium is as follows: during growth, dermatophytes use protein peptides as nutrients, producing alkaline metabolites, and due to the pH indicator included in DTM, the color of the medium changes. Contaminant mold fungi, which may present in the sample, most often use carbohydrates as an energy source without alkalizing the medium.

According to the literature, the diagnostic efficiency of DTM-type media in veterinary practice ranges from 80 to 100% [3, 8, 9, 10]. Efficiency can depend on a number of factors: on the amount of sample material, temperature of incubation, and humidity (since media can dry out quickly). An important factor is the surface area of the medium and its volume. It should be convenient for sowing in various ways and have a supply of nutrients for long-term cultivation of fungi.

It should be remembered that false positive changes in the color of the medium are possible. Dermatophyte colonies should be white or cream in color, while molds are usually colored differently. Using these two characteristics (color of the fungus colony and the change in color of the medium), dermatophytes growth can be accurately confirmed. In addition, microscopy of the resulting colonies can be used. Kaufmann et al. showed the dependence of the efficiency of DTM on microscopy of grown cultures – when the diagnosis was made only by color change, the medium efficiency was 17% lower than with an additional study of colony morphology [11]. However, not all laboratories, and even more
so veterinary clinics, are able to carry out microscopic verification of the culture. Therefore, microscopy of culture is a desirable but not necessary test.

The acidity of nutrient media is one of the important parameters affecting the growth of dermatophytes [12, 13]. The optimal lower pH limit is 5.0-5.5 [14]. With the growth of dermatophytes, the pH shifts to the alkaline side and reaches 8.0-9.0. Within this range, only a limited number of indicator types can be used to record pH changes. In the classic formulation of DTM media, Taplin et al. used phenol red, color change occurs at pH shift >6.8, and the initial pH of the medium is 5.5. A group of scientists from China suggested using bromothymol blue as an indicator, since it has an earlier color shift that occurs at pH = 6.0 [4].

Considering the above, our team set a goal to study the effect of the initial pH and carbohydrate composition (glucose, mannose, mannitol) on the growth and indicator properties of the Russian DTM-Expert medium, which we developed earlier [15].

2. Materials and methods

Culture media: 9 experimental options of the Culture medium DTM-Expert including various combinations of components (I-IX) were prepared. The data are presented in Table 1. Experimental samples of the medium were prepared by dissolving the main components (carbohydrate, peptone, indicator, agar-agar) in water, followed by autoclaving at 121°C for 15 minutes. Selective supplements were aseptically introduced after the medium cooled down to 50-60°C. The pH was adjusted using HCl and NaOH solutions.

Table 1. Quantitative and qualitative composition of the studied options of the DTM-Expert medium, per 1 liter

| Component          | Option I | Option II | Option III | Option IV | Option V | Option VI | Option VII | Option VIII | Option IX |
|--------------------|----------|-----------|------------|-----------|----------|-----------|------------|-------------|----------|
| Mannose            | -        | 10 g      | -          | -         | -        | -         | -          | -           | -        |
| Mannitol           | -        | -         | 10 g       | -         | 5 g      | 10 g      | 10 g       | 5 g         | -        |
| Glucose            | 10 g     | -         | -          | 10 g      | 5 g      | 10 g      | -          | 5 g         | 10 g     |
| Peptone (meat)     | 10 g     | 10 g      | 10 g       | 10 g      | 10 g     | 10 g      | 10 g       | 10 g        | 10 g     |
| Phenol red         | 0.2 g    | 0.2 g     | 0.2 g      | 0.2 g     | 0.2 g    | 0.2 g     | 0.2 g      | -           | 0.025    |
| Bromothymol blue   | -        | -         | -          | -         | -        | -         | -          | 0.025       |          |
| Agar-agar          | 18 g     | 18 g      | 18 g       | 18 g      | 18 g     | 18 g      | 18 g       | 18 g        | 18 g     |
| pH                 | 5.5      | 4.8       | 4.8        | 4.8       | 4.8      | 4.8       | 4.8        | 4.8         | 5.5      |
| Cyclogeximide      | -        | -         | -          | -         | -        | -         | 0.5 g      | 0.5 g       | -        |
| Enrofloxacin       | -        | -         | -          | -         | -        | -         | 0.1 g      | 0.1 g       | -        |

Sabouraud medium with chloramphenicol (HiMedia Laboratories Pvt Ltd) and DTM medium (Dermatophyte Test Medium) were used as a comparative control (HiMedia Laboratories Pvt Ltd).

Fungal strains from the All-Russian state collection of pathogenic and vaccine strains of veterinary important microorganisms of the Federal State Budgetary Scientific Institution “Federal Scientific Centre VIEV” were used as control cultures: Microsporum canis “FL 79-18 Viev”, Trichophyton mentagrophytes “CN 38-18 Viev”, Aspergillus niger “GB 47-19 Viev”, Penicillium chrysogenum “PL 11-19 Viev”, field isolates of Microsporum canis.

Clinical samples hair samples were taken from small domestic animals (cats and dogs) suspected of dermatophytosis.
Equipment: during the experiment we used pH meter HANNA HI 2211-02, laboratory weighing scales VM-512M-II, automatic pipettes, an autoclave (steam sterilizer VK-75), a laminar flow box, laboratory incubator, and a laboratory light microscope.

3. Study design
First stage: control strains of fungi were inoculated on I and IX options of the medium.
Second stage: control strains were inoculated on mediums options II, III, and IV.
Third stage: control strains were inoculated on V and VI options of the medium.
Fourth stage: clinical samples from 54 animals was inoculated on the VII, VIII options of the medium and on DTM (HiMedia).
The inoculations were incubated at a temperature of 28°C, checked every day for the presence of growth and for the color change of the medium for 10 days (within 3 weeks for the inoculations of clinical material).

4. Results and discussion
First stage. The resulting medium, corresponding to option I, at pH = 5.5, had an initial reddish-brown color, which subsequently made it difficult to assess the color change of the medium during dermatophytes growth. At the same time, there were no delays in the growth rate of the control strains. It was found that the color change with the growth of M. canis occurred on the third day, with the growth of Tr. mentagrophytes on the second day (Fig. 1 and 2).

The color change of the medium option IX (indicator – bromothymol blue) during the growth of the control strain M. canis occurred on the 5th day and became saturated only on the 6th day. As Tr. mentagrophytes medium began to change color already on the 2nd day, on the third it became more pronounced (Fig. 3 and 4).
At the second stage, it was decided to decrease the pH value of the experimental media for better visualization of the color change. In the growth process of dermatophytes control strains in the option II, color change was noted on day 4 for M. canis and on day 3 for Tr. mentagrophytes, on the option III the medium changed color on day 2 of M. canis growth and on day 1 for Tr. mentagrophytes. The growth of the control mold strains also changed the medium color on day 6 for Penicillium chrysogenum and on day 7 for Aspergillus niger. The medium with glucose (option IV) changed color on days 3 and 2 with the growth of M. canis and Tr. mentagrophytes, respectively.

At the third stage, combination of mannitol and glucose in different proportions was studied. It was found that the option V of the medium gives better indication results, in comparison with the option VI – the color change with the growth of M. canis occurs 1 day earlier. The color change during the growth of molds also occurred 3-4 days later in the option V, compared with the option VI.

The results for the first, second and third stages are presented in Table 2.

**Table 2.** Terms of color change of the DTM-Expert medium during the growth of control strains (days).

| Fungus strains                      | Option I | Option II | Option III | Option IV | Option V | Option VI | Option IX |
|-------------------------------------|----------|-----------|------------|-----------|----------|-----------|-----------|
| Trichophyton                        | 2        | 3         | 1          | 2         | 2        | 3         | 2         |
| mentagrophytes                      |          |           |            |           |          |           |           |
| Microsporum canis                   | 3        | 4         | 2          | 3         | 3        | 4         | 6         |
| Aspergillus niger                   | -        | -         | 7          | -         | 10       | 6         | -         |
| Penicillium chrysogenum             | -        | -         | 6          | -         | 8        | 5         | 7         |

For the fourth stage – testing on clinical material – two options of the medium were chosen – options III and V, which showed good indicator qualities. They were supplemented with inhibitory and selective additives; on the obtained options of the medium (VII and VIII, respectively), clinical material from small domestic animals suspected of dermatophytosis was inoculated. DTM manufactured by HiMedia was used as a control medium. The results are shown in Table 3.

**Table 3.** The results of clinical material inoculation on DTM-type media.

| No. | Fungal species | Visible growth | Reddening of the medium | Visible growth | Reddening of the medium | Visible growth | Reddening of the medium |
|-----|----------------|----------------|-------------------------|----------------|-------------------------|----------------|-------------------------|
| 1.  | Microsporum sp | 3              | 13                      | 4              | 16                      | 4              | 10                      |
| 2.  | M. canis       | -              | -                       | -              | -                       | -              | -                       |
| 3.  | Microsporum sp | -              | -                       | 4              | 9                       | 5              | 10                      |
| 4.  | M. canis       | 2              | 10                      | 3              | 7                       | 3              | 9                       |
| 5.  | M. canis       | 2              | 9                       | 2              | 4                       | 2              | 4                       |
| 6.  | M. canis       | 2              | 9                       | 3              | 4                       | 3              | 5                       |
| 7.  | M. canis       | 3              | 7                       | 3              | 7                       | 5              | 7                       |
| 8.  | M. canis       | 3              | 8                       | 4              | 7                       | 4              | 7                       |
| 9.  | M. canis       | 2              | 13                      | 3              | 5                       | 3              | 7                       |
| 10. | M. canis       | 2              | 4                       | 3              | 4                       | 3              | 5                       |
| 11. | M. canis       | 5              | 16                      | 5              | 17                      | 7              | 21                      |
| 12. | M. canis       | 7              | 18                      | -              | -                       | 9              | 22                      |
| 13. | M. canis       | 3              | 15                      | 3              | 9                       | 3              | 15                      |
Among 54 animals, 15 were positive for dermatophytes. The samples with a negative inoculation result are not shown in the table. The option VII of the medium made it possible to identify the pathogen in all positive samples, while DTM HiMedia and the option VII identified the pathogen in 13 (86.6%) of 15 cases. On average, the reddening rate of the option VIII after inoculation was 10 days, of the option VII – 8 days, of DTM HiMedia – 11. The reddening rate of the DTM HiMedia after germination was 7 days, of the option VII – 4.6, of the option VIII – 6.2. Also, in all three test media, false positive redness was noted for the growth of molds, most often on Alternaria sp. Of the 54 samples, 3 (5.56%) false positive cases were detected on the medium VIII, redness occurred on average on day 7, on the medium VII – 2 (3.7%) cases, redness on average on day 3, on the medium HiMedia – 5 (9.2%) cases, redness on the 7th day.

This study was continued based on the results of previous studies on the development of Russian DTM-type medium. At the previous stages, we found that meat peptone is the most optimal in terms of growth characteristics, and mannitol and mannose are the optimal carbohydrates [15].

In this study, it was possible to establish that mannitol significantly accelerates the color indication of fungal growth, but not only dermatophytes, but also for contaminating molds. However, when combined with glucose, the medium color changes during the molds’ growth much later than on the medium with mannitol alone, and when dermatophytes grow earlier, compared to the medium with glucose. Thus, we elaborated a balanced DTM medium with early detection of dermatophytes, which corresponds to the option VIII of the media. At the same time, on the experimental medium “DTM-Express”, the least number of false positive results was noted in comparison with the control medium.

The addition of inhibitory supplements, such as cycloheximide, strongly inhibits the growth of contaminant fungi, preventing them from developing into a large colony. As practice shows, it is the large colonies of mold that cause the color change of the medium. Unfortunately, it is extremely difficult to completely get rid of false positive results during the growth of contaminant fungi. Therefore, it is important to use inhibitors to slow down the growth and development of molds. In addition, all molds in the study that changed the medium color had off-white colonies, which indicates that they belonged to the contaminants.

In all studied media options, the species Tr. mentagrophytes changed the color of the medium for 1-3 days of growth, regardless of its composition. Considering this, it is necessary to use several control strains belonging to the genus Microsporum to test media.

The option IX of the medium was made according to a similar recipe presented by Li X et al. [4]. According to our results, the color change of the medium during the growth of the control M. canis strain occurred only on the 5th day, while the medium acquired an intense color by the 7th day. Since the color change occurred much later than on other options of the experimental media, it was decided not to continue working with the option IX.

Dermatophytes grew equally well both at pH = 4.7 and at pH = 5.5. When choosing the initial pH level, we were limited in the choice of indicators that could cover the optimal range for dermatophyte growth, which is 5-8. Therefore, it was decided to use the classic version of indicator – phenol red, and to set the initial pH level to 4.8. At this pH level, the medium is light-orange, almost yellow, making it easier to detect color changes.

In general, the diagnostic efficiency of the option VIII of the medium was 100%, DTM HiMedia and the option VII – 87%. The average reddening rate of the option VIII was 10 days, DTM HiMedia – 11
days, the option VII – 8 days. Thus, the medium “DTM-Expert”, corresponding to the option VIII, promotes quick and accurate indication of dermatophytic fungi in veterinary samples.

The medium does not require special laboratory conditions for use and can be used not only in laboratories, but also in veterinary clinics, which will significantly reduce the cost and speed up the diagnosis of the dermatophytosis.

Acknowledgement
The reported study was funded by Russian Foundation for Basic Research (RFBR), project number 19-316-9006.

References
[1] Taplin D, Zaias N, Rebell G, Blank H 1969 Isolation and Recognition of Dermatophytes on a New Medium (DTM) Arch Dermatol 99(2) 203-9
[2] Debnath C, Mitra T, Kumar A, Samanta I 2016 Detection of dermatophytes in healthy companion dogs and cats in eastern India Iran J Vet Res 17(1) 20-4
[3] Moriello K A 1991 Fungal flora of the haircoat of cats with and without dermatophytosis Med Mycol 29(5) 285-92
[4] Li X F, Shen Y N, Chen W, Chen H, Lv G X, Liu W Da 2009 A new medium for diagnosis of dermatophyte infection Eur J Dermatology 19(1) 34-7
[5] Moriello K A, Kunkle G, Deboer D J 1994 Isolation of Dermatophytes from the Haircoats of Stray Cats from Selected Animal Shelters in two Different Geographic Regions in the United States Vet Dermatol 5(2) 57-62
[6] Alpun G, Ozgur N 2009 Mycological examination of Microsporum canis infection in suspected dermatophytosis of owned and ownerless cats and its asymptomatic carriage Journal of animal and veterinary advances 8(4) 803-6
[7] Gordon E, Idle A, DeTar L 2020 Descriptive epidemiology of companion animal dermatophytosis in a Canadian Pacific Northwest animal shelter system Can Vet J 61(7) 763-70
[8] Singh S, Beena P M 2003 Comparative study of different microscopic techniques and culture media for the isolation of dermatophytes Indian J Med Microbiol 21(1) 21-4
[9] Crozier W J 1977 Dermatophyte Test Medium—An Evaluation of DTM for Diagnosis of Dermatophytosis Australas J Dermatol 5(1) 39-41
[10] Carroll H F 1974 Evaluation of dermatophyte test medium for diagnosis of dermatophytosis Journal of the American Veterinary Medical Association 165(2) 192-5
[11] Kaufmann R, Blum S E, Elad D, Zur G 2016 Comparison between point-of-care dermatophyte test medium and mycology laboratory culture for diagnosis of dermatophytosis in dogs and cats Vet Dermatol 27(4) 284-8
[12] Steindorff A S, Persinoti G F, Monteiro V N, Silva R N 2015 Fungal metabolic diversity Fungal Biopolymolecules 239-62
[13] Martinez-Rossi N M, Persinoti G F, Peres N T A, Rossi A 2012 Role of pH in the pathogenesis of dermatophytes Mycoses 55(5) 381-7
[14] Kadhim S K, Al-Janabi J K, Al-Hamadani A H 2015 In vitro, determination of optimal conditions of growth and proteolytic activity of clinical isolates of Trichophyton rubrum J of Contemporary Medical Sciences 1(3) 9-19
[15] Savinov V A, Ovchinnikov R S, Kapustin A V, Gainullina A A 2019 Express diagnosis of animal dermatophytosis Agrarian science (10) 20-4