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Investigation of the Effectiveness of Chromogenic Media in the Isolation of *Pasteurella multocida* and *Mannheimia haemolytica* from Calf Nasal Swab Samples

Buzağı Nasal Svap Örneklerinden *Pasteurella multocida* ve *Mannheimia haemolytica* İzolasyonunda Kromojenik Besiyerinin Etkinliğinin Araştırılması

Osman Yaşar TEL[1]
Songül ÖTKÜN[2]
Ayfer Güllü YÜCETEPE[1]
Oktay KESKİN[1]

[1]Departmant of Veterinary Microbiology, Harran University, Faculty of Veterinary Medicine, Şanlıurfa, Turkey
[2]Departmant of Veterinary Microbiology, Siirt University, Faculty of Veterinary Medicine, Siirt, Turkey

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Corresponding author/Sorumlu Yazar: Songül ÖTKÜN
E-mail: songulotkun@yahoo.com

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ABSTRACT

Accurate and rapid diagnosis of disease agents is the most important step in terms of control practices. Chromogenic media are culture media that allow the formation of colonies in colors specific to target microorganisms. Because they are target-specific, they do not require validation of results but provide ease of use and time savings. In this study, it was aimed to evaluate the effectiveness of a chromogenic medium (Pasteurella BDR kit) in the detection of *Pasteurella multocida* and *Mannheimia haemolytica* agents in the *Pasteurelleceae* family, which cause respiratory disease in cattle. In this study, nasal swab samples taken from calves showing symptoms of respiratory disease were cultured in chromogenic and standard media. Suspicious growing colonies were confirmed by polymerase chain reaction for *P. multocida* and *M. haemolytica*. While 31 (36.9%) samples formed colonies with the chromogenic medium in the color specific to the target bacteria, 28 (33.3%) samples were positively determined for the target bacteria using the standard cultural method. The results of 26 samples were positive by both cultural diagnosis methods. When the results were compared with the traditional cultural diagnosis, agreement was found to be 92.86%. All colored colonies grown on the chromogenic medium were also tested by polymerase chain reaction (PCR). It was determined that the chromogenic medium detected *P. multocida* at a rate of 92.86% (n = 26) and *M. haemolytica* at a rate of 100% (n = 2) by forming colonies with a family-specific color. As a result, it was concluded that the use of chromogenic media is beneficial in the practical, rapid, and high-accuracy diagnosis of target agents.

Keywords: Chromogenic medium, Pasteurella, bovine respiratory disease, culture

ÖZ

Hastalık etkenlerinin doğru ve hızlı teşhisi, kontrol uygulamaları açısından en önemli basamaktır. Kromojenik besiyerleri hedef mikroorganizmalara özgü renkte kolonilerin meydana gelmesini sağlayan kültür ortamlarıdır. Bunlar hedefe özgü olduklarından sonuçların doğrulama ihtiyacı duymamakla birlikte, kullanım kolaylığı ve zaman tasarrufu sağlarlar. Bu çalışmada, sığırlarda solumun hastalığına neden olan *Pasteurelleceae* ailesinde bulunan *Pasteurella multocida* ve *Mannheimia haemolytica* etkenlerinin tespitinde, kromojenik besiyerinin (Pasteurella BRD Kit) etkinliğini değerlendirmek amaçlandı. Çalışmada solunum hastalığı belirtisi gösteren buzağılardan alınan nasal svap örnekleri kromojenik ve standart besiyerlerinde kültüre edildi. Üreyen şüpheli koloniler *Pasteurella multocida* ve *Mannheimia haemolytica* yönünden Polimeraz Zincir Reaksiyonu (PCR) ile doğrulandı. Kromojenik besiyeri ile 31 (%36,9) numune, hedef bakterilere özgü renkte koloni oluştururken, standard kültürel yöntem ile 28 (%33,3) numune hedef bakteriler yönünden pozitif olarak belirlendi. Her iki kültürel tanı yöntemiyle 26 numunenin sonuçları *pozitif* olarak tespit edildi. Sonuçlar geleneksel kültürel tanı ile karşılaştırıldığında %92,86 oranında uyumlu sonuç

bulundu. Kromojenik besiyerinde üreyen renkli kolonilerin tamamı PCR ile de test edildi. Kromojenik besiyeri *P. multocida*'yı %92,86 (n:26), *M. haemolytica*'yı %100 (n:2) oranında aileye özgü renkte koloni meydana getirme suretiyle tespit ettiği belirlendi.

Sonuç olarak, hedef etkenin pratik, hızlı ve yüksek doğrulukla teşhisinde kromojenik besiyerinin kullanımının yararlı olduğu kanısına varıldı.

Anahtar Kelimeler: Kromojenik besiyeri, *Pasteurella*, sığır solunum hastalığı, kültür

INTRODUCTION

Following the rapid and accurate diagnosis of microorganisms causing disease in animals, the implementation of an appropriate control program is important in many ways, such as preventing epidemics, protecting animal welfare, and reducing antimicrobial resistance and economic losses affecting animal health and welfare worldwide.[1-3] The etiology of the disease, which is an important cause of death in cattle, is multifactoral, and bacterial agents such as *Pasteurella multocida, Mannheimia haemolytica*, and *Mycoplasma bovis* act as opportunistic pathogens and adversely affect the prognosis, especially in calves.[4,6] The diagnosis of bovine respiratory tract disease is made by cultural, molecular, or serological examination of samples taken from the animal.[7,8] These diagnostic methods are widely used and play an important role in the investigation of the disease,[2,3,9-11] but these methods are time-consuming and require a well-equipped laboratory and experienced personnel.

In recent years, highly specific chromogenic media targeting pathogenic or resistant microorganisms have been developed for rapid diagnosis and treatment of diseases.[12-14] These media make it possible to distinguish microorganisms according to colony color, as they contain chromogenic substrates that allow the formation of colonies with a color specific to the target microorganisms. Compared to standard methods, these culture media are easy to use and the results are easy to read, and the need for confirmation testing is less. In addition, chromogenic media provides cost and time savings with high accuracy and sensitivity, as well as fast and practical identification in samples where contamination is possible.[12,15-17] The performance of chromogenic media has been evaluated in many studies, such as mastitis, urinary tract pathogens, and the diagnosis of resistant microorganisms.[18-20]

The aim of this study was to compare the performance of chromogenic media in the isolation of *P. multocida* and *M. haemolytica* with the standard cultural diagnosis method using nasal swab samples from calves with respiratory tract disease.

MATERIAL AND METHODS

The study was carried out with samples brought to our laboratory for diagnosis purposes. Nasal swab samples were taken from 84 calves with the suspicion of respiratory disease outbreak, such as difficulty in breathing and coughing among young animals on a farm. Samples in transport medium and cold chain were delivered to the Microbiology Department laboratory.

Microbiological Culture

Standard microbiological methods were applied to the samples in the laboratory for microbiological analysis. In summary, 84 nasal swab specimens were seeded on 5% sheep blood agar and MacConkey agar (Merck, Germany) and incubated aerobically at 37°C for 24-48 hours. The growing microorganisms were evaluated in terms of hemolysis features, Gram staining, and other biochemical features and their identifications were made.[21]

The samples were simultaneously cultivated of a selective and chromogenic medium (Pasteurella BRD Kit, Arbilim Biyoteknoloji, Türkiye) developed to facilitate the detection of *Pasteurellaceae* and incubated at 37°C for 24-48 hours under aerobic conditions. On chromogenic media, members of the *Pasteurellaceae* family form colonies ranging in color from pink to lilac, while inhibiting coliforms form blue-steel-blue colonies. Other Gram (–) bacteria are inhibited or form colorless colonies. The growth of yeasts and Gram (+) microorganisms is suppressed.

Molecular Diagnosis

DNA Extraction

The boiling method was used for DNA extraction. Suspected bacteria were incubated aerobically overnight at 37°C in a brain heart infusion medium (Merck), and 2 mL of bacterial suspension was incubated at –20°C for 10 minutes. Then, the thawed suspension was centrifuged and the pellet was homogenized with 200 µL of ddH_2O and boiled in a water bath for 10 minutes, and then the centrifugation was repeated and the supernatant was stored at 4°C to be used for polymerase chain reactions (PCRs).[22]

Polymerase Chain Reaction Step

The reaction was performed according to the method reported by Deressa et al.[23] comprising a PCR mix, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$), 0.2 mM dNTP, 0.5 mM *P. multocida* primers and 2 mM *M. haemolytica* primer, 2 units of Taq polymerase and then the final product was prepared in a volume of 25 µL using 1 µL of template DNA as a sample (Table 1).

Table 1. Primers Used in PCR and Length of Amplification Product (Base pair)

Target Bacteria	*Pasteurella multocida*	*Mannheimia haemolytica*
Oligonucleotides (5'-3')	GCTGTAAACGAACTCGCCC ATCCGCTATTTACCCAGTGG	GTTTGTAAGATATCCCATTT CGTTTTCCACTTGCGTGA
Amplicon length	460 bp	1022 bp

The PCR amplification consisted of 30 cycles of 1 minute at 95°C, 1 minute at 48°C, and 30 seconds at 72°C, followed by 3 minutes of pre-denaturation at 95°C.

In order to evaluate the PCR products, the presence of specific bands of 460 bp for *P. multocida* and 1022 bp for *M. haemolytica* was investigated by running it on 2% agarose gel and staining it with ethidium bromide.

RESULTS

In this study, the effectiveness of chromogenic medium in the isolation of *P. multocida* and *M. haemolytica* from nasal swab samples taken from young animals with respiratory problems was determined by comparing them with standard diagnostic methods.

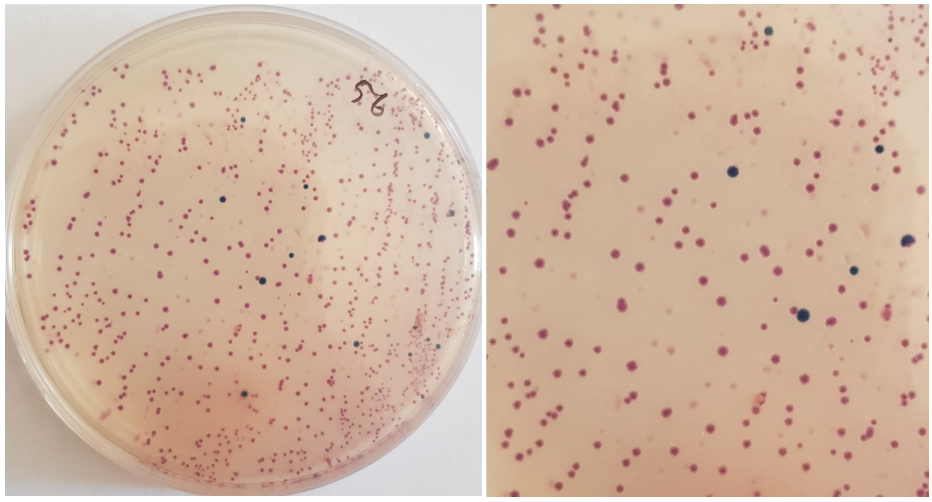

Figure 1. Colony morphology of bacteria in Pasteurella BRD Kit. Pink-mauve colonies: Pasteurellaceae, blue-steel-blue colonies: Coliforms.

All of the 84 nasal swab samples from calves were seeded on standard media and chromogenic agar simultaneously. A history of antibiotic treatment was obtained in 16 (19.05%) of the sampled calves. Although bacterial growth was observed in the blood agar medium in all of the samples, growth was observed in 14 (16.66%) samples on the MacConkey agar medium. While 2 of the growing bacteria were identified as *M. haemolytica*, 28 (33.3%) of the suspicious colonies grown on blood agar were found to be positive for *P. multocida* according to standard identification methods (Table 2). *M. haemolytica*-isolated samples (n = 2) were also positive for *P. multocida,* which was also isolated and identified from 2 of the samples (n = 16) taken from animals that were started on antibiotic treatment.

A total of 31 (36.9%) specimens formed colonies (pink-lilac in color) specific to the *Pasteurellaceae* family in cultivation on chromogenic medium (Figure 1). In the evaluation, 4 (4.76%) samples were found to be suspicious and there were 49 (58.33%) negative samples (Table 2). Moreover, 5 samples found positive in the chromogenic medium were found to be negative by standard methods, 1 sample that was found to be positive by standard methods was found to be negative in the chromogenic medium, and 1 sample was suspicious. Of the samples, 30.95% (26) were found positive with the same results in standard and chromogenic media, and the agreement between the standard identification and chromogenic media was 92.86% (Table 2).

Bacteria found to be positive and suspicious by both cultural methods (62 in total) were molecularly analyzed for *M. haemolytica* and *P. multocida* by PCR. The PCR results were fully compatible with the standard method. It was found that 28 (33.3%) samples formed bands compatible with *P. multocida* (Figure 2). Of these, 26 were positive, 1 was negative, and 1 was suspicious on chromogenic medium. Moreover, 2 samples were culturally and molecularly identified as *M. haemolytica*, (Figure 3) and they were also positive for *P. multocida*. It was found that 5 of the PCR results were negative for *P. multocida* and *M. haemolytica*, which produced a color specific to the *Pasteurellaceae* family in chromogenic culture.

DISCUSSION

The animal and human upper respiratory tracts are home to a wide variety of potentially pathogenic commensal microorganisms that are in competition with each other. Among these microorganisms, which are defined as pathobionts, members of the *Pasteurellaceae* family are commonly found.[24,25] There are many pathogenic species in this family, and *P. multocida* and *M. haemolytica* cause fatal pneumonia, especially in calves younger than 6 months old and under stress.[24,26,27] In this study, the diagnosis performance of chromogenic medium developed to detect bacteria in the *Pasteurellaceae* family was tested and compared with the standard culture method. When the results were compared, it was seen that there was a high rate of agreement (92.86%).

Payne and Roscoe[19] compared the performance of 2 different commercial chromogenic agars (chromID CPS and UriSelect), which detect many important pathogens, such as *Escherichia coli, Proteus mirabilis, and Enterococcus spp.*, in clinical urine samples (human) with standard culture at rates of 93% and 93.1%, and obtained consistent results. In the current study, in which the performance of the chromogenic medium developed for the detection of agents in the *Pasteurellaceae* family was compared with the traditional culture, the compatibility in the diagnosis of nasal swab samples was 92.86%. Cole et al[28] found 96.6% compatible results in their study where they compared cat and dog urine samples with traditional culture using choromogenic triplates (UTid+), which was developed for dairy culture purposes and allows for the identification of many microorganisms, such as *E. coli, Staphylococcus* spp., and *Enterococcus* spp. When the

Table 2. Results of the Pasteurella BRD Kit and Standard Cultural Diagnosis

		Standard Culture Result		
		Pasteurella	*Pasteurella* Negative	Total
Pasteurella Kit growth result	Positive count/percentage	26/30.95%	5/5.95%	31/36.9%
	Negative count/percentage	1/1.19%	48/57.14%	49/58.33%
	Suspect count/percentage	1/1.19%	3/3.57%	4/4.76%
	Total/percentage	28/33.33%	56/66.66%	84/100%

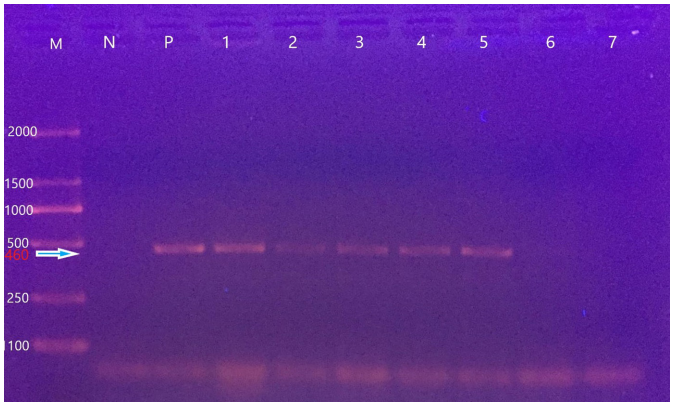

Figure 2. *Pasteurella multocida* polymerase chain reaction results: M, ladder; N, negative control; P, positive control; 1-5, positive samples; 6 and 7: negative samples.

results were compared, despite the clinically compatible results, it was thought that the small differences could have been caused by many variables, such as the chromogenic environment and target microorganisms, sampling method, researcher, and laboratory. The performance of chromogenic media in rapid microbiological identification of mastitis has been evaluated in many studies and its importance in diagnosis has been demonstrated.[17,18]

The number of studies investigating the effectiveness of chromogenic culture media in the detection of agents in respiratory system diseases is limited.[29] As a result of culturing 84 nasal swab specimens in this study in chromogenic media, 31 (36.9%) specimens formed a pink-lilac-colored colony specific to the *Pasteurellaceae* family (Figure 1). The results were consistent with traditional cultural diagnosis and PCR. Many studies[18,28,30] have concluded that, similar to the results of this study, chromogenic environments will play an important role in clinical diagnosis in terms of rapid screening of pathogens.

P. multocida and *M. haemolytica* are frequently isolated bacterial agents in pneumonia.[31] Since the role of PCR in the accurate and rapid detection of these agents is undeniable,[32] suspicious bacteria were examined by PCR for the presence of *P. multocida* and *M. haemolytica*. It was found that 26 samples were positive for *P. multocida* (Figure 2) and 2 samples were positive for *P. multocida* and *M. haemolytica*. In these studies, the isolation rates of the 2 pathogens were found in different numbers in healthy animals and animals showing respiratory disease symptoms, and

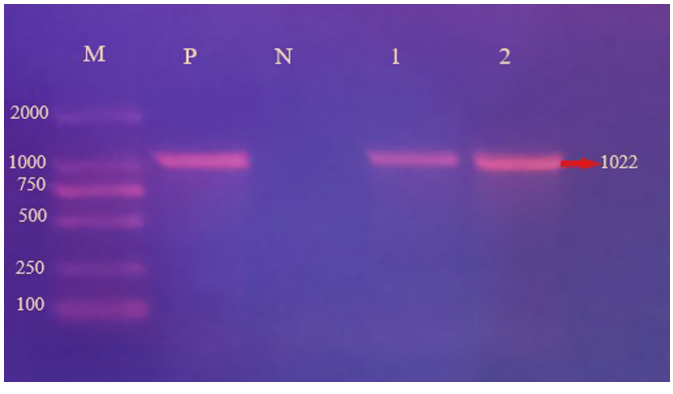

Figure 3. *Mannheimia haemolytica* polymerase chain reaction result. M, ladder; P, positive control; N, negative control; 1 and 2, positive samples.

the complex etiological situation played a big role in this. However, these 2 bacterial agents were found to be significantly lower in the healthy animals when compared to the sick animals.[31,33-35] Tel and Keskin[36] isolated and identified *M. haemolytica* at a rate of 12.5% and *P. multocida* at a rate of 31.6% in their study with 240 pneumonic lung samples. In the current study, *P. multocida* was found at a rate of 33.3% and *M. haemolytica* was found at a rate of 2.38% by PCR from 84 calves showing respiratory system disease. Although the isolation rate of *P. multocida* was close to the findings of Tel and Keskin,[36] the difference in the rate of *M. haemolytica* was thought to be related to variables such as age, geographical region, and sampling time (Figure 3).

One of the important aims of this study was to test the performance of the chromogenic medium in detecting *P. multocida* and *M. haemolytica*. Of the 31 samples that formed pink-mauve colonies specific to the *Pasteurelleceae* family on chromogenic media, 26 were identified as *P. multocida* and 2 were identified as *M. haemolytica* by PCR. One of the 2 samples identified as *P. multocida* by PCR was identified as negative in the chromogenic medium, and in the other, it was identified as suspicious. Based on these data, it was observed that the chromogenic medium formed colonies with a family-specific color at a high rate of 92.86% (n = 26) for *P. multocida* and 100% (n = 2) for *M. haemolytica*.

There are many bacterial genera and species in the *Pasteurellaceae* family.[37] Of the samples, 5 that formed colonies with a color specific to the *Pasteurellaceae* family on chromogenic media were found to be negative for *P. multocida* and *M. haemolytica* by PCR, and no evaluation was made in terms of other possible factors.

As a result, chromogenic medium was used to detect *P. multocida* and *M. haemolytica* from the nasal swabs of calves with high accuracy. In the preparation and visual evaluation and reading of the results, the use of chromogenic medium, which does not require special training and gives results in a short time, was found to be advantageous. Farms will benefit from accurate and rapid diagnosis and treatment in places where contamination is highly likely, which do not have equipped laboratories such as clinics and sufficient specialized personnel. In addition, it has been predicted that the use of such media will play an important role in preventing unnecessary drug use and antimicrobial resistance.

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