

# Accuracy of 12h-Petrifilm™-plates as a rapid on-farm test for evidence-based mastitis therapy on a dairy farm in Germany

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## Abstract

Antibiotic resistance is a highly discussed issue in society. The use of antibiotics in livestock husbandry is critically viewed. As a result, the European commission issued guidelines for the prudent use of antimicrobial agents in veterinary medicine in 2015 (EU 2015/C 299/04). Thus, alternative approaches for treating cows in the future are necessary. In particular, the treatment of mastitis, the most frequent disease in the dairy industry, causes a high use of antibiotics. Implementing an on-farm test to assign mastitis pathogens to classes of pathogens (Gram-positive, Gram-negative, no bacterial growth) before treatment decisions are made provides the basis for an evidence-based mastitis therapy concept. Rapid Aerobic Count plates and Rapid Coliform Count plates of 3M™ Petrifilm™ (3M™ Neuss, Germany) were used in combination as a 12h rapid on-farm test concept. The diagnostic sensitivity and specificity were evaluated in comparison to the standard laboratory examination. 129 mastitis milk samples from the quarters with clinical mastitis by a conventional dairy farm in Germany were used for evaluation. Results were examined 12 hours after inoculating the Petrifilm™-plates. The sensitivity for Gram-positive pathogens was 93.2%. For Gram-negative pathogens it was 88.9%. The specificity was 39.0% for Gram-positive pathogens and 97.5% for Gram-negative pathogens, respectively. To get good results, training the milking personnel in taking aseptic milk samples as well as inoculating and evaluating the test is inevitable. On the basis of the results the 12h-Petrifilm™ concept can serve as a basis for treatment decisions in the evidence-based mastitis therapy in dairy herds with a low percentage of infections with eukaryotic pathogens and can complement bacteriological culture.

**Keywords:** dairy cow, clinical mastitis, rapid on-farm test, evidence-based mastitis therapy

## Introduction

The bovine mastitis is one of the most frequent and costly diseases in dairy cattle and is often treated with antibiotics [1, 2, 3]. Nowadays, due to public concerns about using antibiotics in livestock husbandry and to avoid antimicrobial resistance, it is important to develop alternative approaches for treating cows in future. Therefore, the European commission recently issued guidelines for the prudent use of antimicrobial agents in veterinary medicine (EU 2015/C 299/04).

The commission advises the restrictive use of antibiotics, which should only be applied for clear indication and treatment necessary to protect the animal. In addition, the commission calls for the use of rapid tests prior to the treatment of clinical mastitis, because a forward-looking responsible use of intramammary antibiotics should be based on cultural results [4].

Some studies [5,6] have shown a high spontaneous cure rate for the mild and moderate clinical mastitis cases caused by Gram-negative pathogens, especially *Escherichia coli*. Thus, clinical mastitis cases showing abnormal milk with or without an infected udder do not benefit from an antibiotic therapy if they are caused by Gram-negative pathogens [6]. Nonetheless, it is recommended to treat clinical mastitis cases caused by Gram-positive pathogens with intramammary antimicrobial applications [6]. A therapy concept where Gram-negative mastitis cases were not given any antibiotic treatment did not differ in cure rates from a therapy where all cases were treated with antimicrobial products, similar to cases without any proof of a pathogen [7]. This confirms the statement by Roberson that the use of local antibiotics is limited to clinical mastitis cases with a Gram-positive test result [6]. Hence, distinguishing between Gram-negative and Gram-positive etiologies can reduce the use of antibiotics when selective treatment is applied [7].

3M™ (Neuss, Germany) established Petrifilm™ plates, which detect the mastitis causing pathogen (MCP) group on-farm [8]. The newly developed Rapid Aerobic Count (RAC) in combination with the Rapid Coliform Count (RCC) Petrifilm™, enables the distinction of Gram-positive MCP, Gram-negative MCP and no bacterial growth [9]. Twelve hours after inoculation, results are available and serve as a basis for an evidence-based mastitis therapy concept. This concept contributes to a reduction in antibiotic usage as well as consistent therapeutic success by only treating cows with Gram-positive test results with intramammary antibiotic compounds [7].

The results of a newly developed 12h-rapid on-farm test (Rapid Aerobic Count (RAC) and Rapid Coliform Count (RCC) of 3M™ Petrifilm™ (3M™, Neuss, Germany)) were evaluated in relation to the conventional standard method, the microbiological culture of the MCP [10, 12]. The aim of the present study was to demonstrate test characteristics on the basis of a field study on a conventional dairy farm.

## Material and Methods

### Farm:

The study was conducted on a conventional dairy farm in Lower-Saxony, Germany, from June 2016 to January 2017. Approximately 900 lactating Holstein-Friesian cows were milked three times a day in a 32-cow rotary milking parlour with an average milk yield of 11,165 kg milk per cow and year (energy corrected milk, **ECM**). The average dairy herd improvement somatic cell count amounted to 390,700 cells/milliliter (mL). The percentage of healthy cows (cows below 100,000 cells/mL) in this period was 35.4% and the new infection rate within lactation was 32.0%. The cows were kept in a free-stall barn, being fed with a total mixed ration depending on the milk yield.

### Mastitis cases:

Clinical mastitis cases were detected by forestripping done by trained milking personnel directly before milking. A clinical mastitis was defined by the appearance of abnormal milk character (clots, blood, water → mastitis grade M1 (low)), possibly with a swollen and/or heated udder (mastitis grade M2 (moderate)), or, in severe cases, accompanied by additional systemic signs of illness (e.g. fever, loss of appetite; mastitis grade M3 (high)) [5]. After detecting clinical mastitis, a quarter sample was aseptically taken.

### Samples:

The quarter samples were drawn into test tubes (13 mL) with a preserving agent containing boric acid (0.5 mL Ly 20) [11]. They were taken according to the regulations of the German Veterinary Association (**GVA**) [10]. After cleaning and discarding a few milk jets the teat ends were disinfected with 70% ethanol and a few jets of milk were milked into the test tube. The milking personnel was trained in detecting clinical mastitis and taking samples. These were stored at 7°C until transportation to the laboratory twice a week. The laboratory personnel was unaware of the results of the on-farm test.

### On-farm analysis with 3M™ Petrifilm™:

Two Petrifilm™ plates (3M™, Neuss, Germany), the Rapid Aerobic Count (RAC) plate and the Rapid Coliform Count (RCC) plate, were used to examine the mastitis milk samples. Milk samples were mixed and diluted 1:10 with sterile Ringer solution (Merck, Darmstadt, Germany). After thorough mixing, the dilution was added to the Petrifilm™ (1mL per plate) and spread out as described in the instruction manual (3M™ Petrifilm™). This procedure was performed twice – once for the RAC and once for the RCC. The plates were incubated for 12 hours at 37°C. According to McCarron et al. [9], five or more colonies on the Aerobic Count Petrifilm™ and 20 or more colonies on the Coliform Count Petrifilm™ were marked as a positive result. This limit value was also selected in this study for RAC and RCC. When both Petrifilm™-plates showed bacterial growth, the result was interpreted as Gram-negative MCP, whereas bacterial growth only on the RAC was detected as Gram-positive MCP. No bacterial growth was characterized on both plates.

### Microbiological analysis:

A microbiological analysis of the mastitis milk samples was performed following the examination standards as described in a study of Mansion-de Vries et al. [8]. The examination followed the regulations of the GVA and the laboratory handbook of the National Mastitis Council (**NMC**) [10, 12]. Laboratory personnel plated ten microliters of a well-mixed quarter foremilk sample with a sterile loop onto the quadrant of an aesculin sheep-blood agar plate (Oxoid, Wesel, Germany). The plates were incubated for 48 hours at 37°C under aerobic conditions and examined twice, 24 and 48 hours after inoculation. The grown colonies were identified by their colony morphology, Gram staining, haemolysis patterns and their aesculin hydrolysis. Additionally, other

biochemical properties like the activity of catalase, clumping factor test, Lancefield serotyping, activity of cytochrome oxidase C and oxidation-fermentation of glucose were considered for further identification. Deviating from the regulations of the German Veterinary Association, a sample was claimed positive for environmental organisms if more than five colonies were identified in the examination (standard operation procedure in the laboratory). As the examinations by Smith indicate, the limit value of ten colony-forming units/0.01mL may be too high regarding coliform pathogens [13]. We aimed to achieve a higher sensitivity because the rapid on-farm test worked with an inoculum of 0.1mL. Withal, already one colony led to a positive result for cow-associated pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Trueperella pyogenes* [10]. If more than two different colony types were detected in one milk sample, the sample was categorized as contaminated. Two different colony types in one sample were referred to as mixed infection.

### Statistical analysis:

The data were collected in Microsoft Access and Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA).

The accuracy of the Petrifilm™ concept was estimated by comparing the results of the rapid test concept with those of a microbiological culture that was considered as the reference test. Therefore, the test characteristics sensitivity, specificity, positive and negative predictive value, Youden's Index and the true and apparent prevalence were calculated. The evaluation was done using a 95% confidence interval (**CI**) by WinEpiscope 2.0 (<http://www.winepi.net/30.08.2017>).

## Results

During the study period, 142 clinical mastitis milk samples from 142

**Table 1: Amount and distribution of mastitis-causing pathogens in 142 mastitis milk samples from clinical mastitis cases (one case per cow) resulting from a six-month study on a dairy farm with 900 lactating cows in Lower-Saxony, Germany (microbiological culture)**

Findings	Amount	Percentage	Confidence interval (95%)
No growth	17	12.0	(10.0; 14.0)
<i>Staphylococcus aureus</i>	19	13.4	(11.3; 15.5)
<i>Streptococcus uberis</i>	24	16.9	(14.6; 19.2)
<i>Prototheca spp.</i>	19	13.4	(11.3; 15.5)
<i>Enterococcus spp.</i>	6	4.2	(3.0; 5.4)
CNS*	6	4.2	(3.0; 5.4)
Coryneform bacteria	3	2.1	(1.2; 3.0)
<i>Bacillus spp.</i>	4	2.8	(1.8; 3.8)
<i>Trueperella pyogenes</i>	2	1.4	(0.7; 2.1)
Group C streptococci	2	1.4	(0.7; 2.1)
<i>Escherichia coli</i>	4	2.8	(1.8; 3.8)
Coliform bacteria	1	0.7	(0.2; 1.2)
Mixed growth <sup>†</sup>	22	15.5	(13.3; 17.7)
Contaminated <sup>‡</sup>	13	9.2	(7.4; 11.0)
<b>Total</b>	<b>142</b>	<b>100</b>	

\*Coagulase negative staphylococci

<sup>†</sup>Mixed growth: two different pathogens in one mastitis milk sample

<sup>‡</sup>Contaminated: more than two different pathogens in one mastitis milk sample

different cows were collected. So, Gram-positive pathogens such as *Staphylococcus aureus* (13.4%; CI: (11.3; 15.5)) or *Streptococcus uberis* (16.9%; (14.6; 19.2)) were identified in the bacteriological culture. Other findings were cases with no microbiological growth (12.0%; (10.0; 14.0)) and *Prototheca spp.* (13.4%; (11.3; 15.5)). In 15.5% (13.3; 17.7) of all samples, two different colony types have been detected. This was referred to as mixed growth. Gram-negative bacteria were only identified in five samples (four *Escherichia coli* 2.8%; (1.8; 3.8); one coliform bacteria 0.7%; (0.2; 1.2)) using the standard method. Further distribution is described in Table 1. For evaluation, 13 cases were excluded from the study because the milk samples were contaminated. Consequently, for 129 cases the results of the rapid test were evaluated and compared with the results of a conventional microbiological culture that served as reference method. Table 2 contains the results of the statistical analysis differentiated according to the MCP. For Gram-positive MCP, the rapid test had a sensitivity of 93.2% (87.9, 98.4) and a specificity of 39.0% (24.1, 54.0). For Gram-negative pathogens, the test system had a sensitivity of 88.9% (68.4, 109.4) and a specificity of 97.5% (94.7, 100.3). The absolute agreement for Gram-positive MCP was 76.0% (kappa coefficient: 0.368, CI (0.208, 0.527)) and 96.9% for Gram-negative pathogens (kappa coefficient: 0.783, CI (0.612, 0.955)). Anyhow, the verification of protothecal infections in this study were inconsistent. In 73.7% of these cases, the rapid test showed an overall Gram-positive result (data not shown).

The evaluation only contains the first case of clinical mastitis in each cow in the current lactation. Subsequent cases were omitted from the evaluation. The rapid test worked the best for severe cases of mastitis (M3) (sensitivity and specificity of 100%).

## Discussion

In this study, the diagnostic certainty and suitability of 3M™ Petrifilm™ plates (3M™, Neuss, Germany) were examined with clinical mastitis milk samples on a conventional dairy farm compared with the standard laboratory examination. Already 12 hours after inoculating the rapid on-farm test, results could be evaluated. The sensitivity for Gram-positive pathogens (93.2%) was similar with the results from previous studies (93.8%, 89.9%, 90.0%) [7, 11, 6]. In contrast to these studies, the specificity for Gram-positive pathogens was described with 39.0% (70.1%, 88.4%) [9, 8]. However, the specificity for Gram-positive pathogens in this study was similar to the results of Gitau et al. (51.0%) [14]. Compared with the standard method, the detection

rate of the rapid test was higher due to the ten-fold higher inoculation volume (0.1 mL sample for Petrifilm™, 0.01 mL sample for the standard method). Thus, more samples could be recognized as pathogen-positive with the rapid test than with the standard method due to a higher amount of bacteria in the initial volume. Yet, the inoculum in the laboratory method is consistent with international standards following the regulations of the GVA and NMC [10, 12] and is seen as gold standard in microbiological examinations. The detection rate of the experimental method comes close to that of the gold standard if the inoculum increases [15]. Nonetheless, with a high number of detected Gram-positive pathogens, the negative predictive value still achieved 72.7%. The results of sensitivity in this study are similar to the results described in a study by Mansion-de Vries et al. (Gram-positive MCP: 89.9, Gram-negative MCP: 85.2, no growth: 41.0) [8].

The study design may have influenced the results of the microbiological analysis. The cold storage of milk samples could have influenced the growth of psychotropic bacteria or could have had a damaging effect on coliform bacteria. The use of a preserving agent could also have influenced the growth in the microbiological culture. Moreover, the limit value for environmental bacteria was decreased to five colonies because the examinations by Smith indicate that the limit value of ten colony-forming units/0.01mL may be too high regarding coliform pathogens [13]. We aimed to achieve a higher sensitivity because the rapid on-farm test worked with an inoculum of 0.1mL. This could have influenced proof of Gram-negative bacteria. A clear classification of protothecal infections with the test system was also not possible because the rapid test showed a Gram-positive result in nearly three quarters of protothecal infections. The rapid test is not useful for detecting protothecal and yeast infections because they do not grow within 12 hours [8]. Attention should be given to the fact that the study was only concerned with the accuracy of the test for clinical mastitis. Therefore, these results are expected to be different for subclinical mastitis.

A disadvantage of the Petrifilm™-method is its inability to discriminate contaminated milk samples from uncontaminated ones or to recognize mixed infections [14]. In cases of mixed growth containing a Gram-positive MCP as well as a Gram-negative MCP, the test system was truly positive for both Gram-negative and Gram-positive bacteria. However, this result could not be differentiated from a Gram-negative test result because both Petrifilm™ plates (RAC and RCC) showed a positive result. Consequently, cows with a false positive Gram-negative result would

**Table 2: Test characteristics of Rapid Aerobic Count and Rapid Coliform Count (3M™ Petrifilm™) describing the correct prediction of the MCP<sup>1</sup> group (Gram-positive, Gram-negative, no bacterial growth) in 129 milk samples taken on a dairy farm with 900 lactating cows in nearly six months**

Test characteristics (95% CI)	Gram-positive MCP [%]	Gram-negative MCP [%]	No bacterial growth [%]
Sensitivity	93.2 (87.9, 98.4) <sup>3</sup>	88.9 (68.4, 109.4) <sup>3</sup>	30.6 (15.5, 45.6) <sup>3</sup>
Specificity	39.0 (24.1, 54.0)	97.5 (94.7, 100.3)	95.7 (91.6, 99.8)
Positive predictive value	76.6 (68.6, 84.7)	72.7 (46.6, 99.0)	73.3 (51.0, 95.7)
Negative predictive value	72.7 (54.1, 91.3)	99.2 (97.5, 100.8)	78.1 (70.5, 85.7)
Youden's index <sup>2</sup>	0.322 (0.164, 0.480)	0.864 (0.657, 1.071)	0.263 (0.107, 0.419)
True prevalence	68.2 (60.2, 76.3)	7.0 (2.6, 11.4)	27.9 (20.2, 35.6)
Apparent prevalence	82.9 (76.5, 89.4)	8.5 (3.7, 13.3)	11.6 (6.1, 17.2)

<sup>1</sup> Mastitis-causing pathogen

<sup>2</sup> Youden's index not in %

<sup>3</sup> 95% confidence interval in brackets

not receive antibiotic therapy although they would benefit from it. This could lead to lower chances of being cured. On the other hand, false Gram-positive results lead to an increased use of antibiotics or rather do not lead to a reduction in antibiotics.

One advantage of the rapid test with the newly developed RAC is that it saves 12 hours of time compared to the 24h-Petrifilm™ concept used in the study by Mansion-de Vries et al. [8]. As described in their study, transporting milk samples to a laboratory is also unnecessary. Thus, results are available within two milking times and provide the basis for an evidence-based mastitis therapy concept. Therapy decisions can be made based on the results and the deferred therapy can commence in a time-frame that is associated with only minimal adverse effects [4]. The test can be used on-farm by the farm personnel because its inoculation and evaluation are easy to handle. After a short briefing, the farm personnel could carry out the test on their own. Before using the test, the disposal of used Petrifilm™-plates should also be organized because they have to be disposed separately from normal residual waste. Used tests have to be decontaminated. Mistakes when evaluating Petrifilm™-plates could also lead to false results and therefore to false therapy decisions resulting in lower cure rates or increased antibiotic use. Therefore, it is very important to train the milking personnel in taking aseptic milk samples as well as inoculating and evaluating the test. Furthermore, there is a risk of failing to recognize certain pathogens like *Prototheca spp.* and yeasts with the rapid test. Thus, regular microbiological examinations of the pathogen spectrum in the herd are inevitable [8]. Moreover, the rapid on-farm test is not able to test for antimicrobial resistance which is commonly performed after cultivating the pathogens in a microbiological culture.

## Conclusions

All in all, the Petrifilm™ concept had a high sensitivity of 93.0% for Gram-positive and 83.3% for Gram-negative pathogens. The specificity for no detected bacterial growth was 94.8%. This leads to the conclusion that the Petrifilm™ concept is an effective tool and thus useful in diagnosing the pathogen group in dairy herds with a low percentage of infections with eukaryotic pathogens and uncontaminated milk samples. The rapid test was incapable of detecting eukaryotic cells and could lead to false results for samples with non-uniformly mixed infections (Gram-positive and Gram-negative pathogens simultaneously) or contaminated samples. Nonetheless, it provides the basis for therapy decisions in addition to the conventional standard method and can be used to support an evidence-based mastitis therapy concept in dairy herds with mainly bacterial pathogens.

## Disclosure of conflicts of interest

All persons involved in this study declare no conflict of interests.

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