# Pyodermie beim Hund: *mecA* bleibt erhalten bei Herstellung autogener Bakterine aus Meticillin-resistentem *Staphylococcus pseudintermedius* (MRSP) und *S. aureus* (MRSA)

# Canine pyoderma: *mecA* persists autogenous bacterin formulation from meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) and *S. aureus* (MRSA)

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## Schlüsselwörter

Antibiotikaresistenz, superfizielle Pyodermie, wiederkehrend, Phenol, Formalin, DNA

## Key words

Antimicrobial resistance, superficial pyoderma, recurrent, phenol, formalin, DNA

## Abstract

**Objective:** Autogenous *Staphylococcus pseudintermedius* bacterins can reduce prescribing of antimicrobials in the management of canine recurrent pyoderma. However, increasing prevalence of meticillin-resistant, *mecA*-positive *S. pseudintermedius* (MRSP) raises concern over dispersal of *mecA* through bacterin therapy. We investigated the presence and integrity of *mecA* in bacterin formulations after manufacturing.

**Material and methods:** Twenty clinical isolates (12 MRSP, 7 MR-*S. aureus*, 1 meticillin-susceptible SP) were investigated. Pellets from overnight growth were washed three times with 0.5% phenol saline, followed by addition of 0.1ml 10% Formal-Saline to 10ml Phenol-Saline. Sterility was confirmed, and DNA extracted using both a standard genomic extraction kit and one recommended for formalin-fixed tissue samples (FFPE). The presence of *mecA* was determined after PCR and its integrity examined in five randomly selected samples after sequencing.

**Results:** In all bacterins from meticillin-resistant isolates, *mecA* was detected following FFPE extraction; products aligned fully to a reported *mecA* sequence. After standard DNA extraction, *mecA* was seen in 16/19 samples.

**Conclusion:** Persistence of *mecA* in MRSP bacterins suggests that dispersal of this important resistance mediator through therapy may be possible. While the ability of skin bacteria to uptake naked DNA remains unclear, it seems prudent to only formulate autogenous bacterins from *mecA*-negative *S. pseudintermedius* to avoid unnecessary spread of *mecA*.

## Zusammenfassung

**Ziel der Studie:** Autogene *Staphylococcus pseudintermedius* Bakterine (Autovakzine) können helfen, die Verschreibung von Antibiotika in der Therapie wiederkehrender Pyodermien des Hundes zu reduzieren. Die Zunahme Meticillin-resistenter, *mecA*-positiver *Staphylococcus pseudintermedius*-Infektionen (MRSP) ist besorgniserregend, da Bakterin-Therapie möglicherweise zur Verbreitung von *mecA* beitragen kann. Wir untersuchten daher das Auftreten und die Integrität von *mecA* in Autovakzinen nach der Herstellung.

**Material und Methoden:** Zwanzig klinische Isolate (12 MRSP, 7 MR-*S. aureus*, 1 Meticillin-empfindlicher SP) wurden untersucht. Zellpellets wurden dreimal mit 0,5%iger Phenol-Kochsalzlösung gewaschen, danach folgte eine Zugabe von 0,1ml 10%iger Formalin-Kochsalzlösung zu 10ml Phenol-Kochsalzlösung. Sterilität wurde bestätigt und DNA mittels eines standardisierten genomischen Extraktionskits sowie eines weiteren für Formalin-fixiertes Gewebe (FFPE) extrahiert. Die Präsenz von *mecA* wurde mit PCR bestimmt und seine Integrität in fünf zufällig ausgewählten Proben nach Sequenzierung untersucht.

**Ergebnisse:** In allen Bakterinen aus Meticillin-resistenten Isolaten wurde *mecA* nach FFPE Extraktion gefunden; die Produkte wurden alle mit einem beschriebenen *mecA* abgeglichen. Nach einer Standard DNA Extraktion wurde *mecA* in 16/19 Proben gefunden.
**Klinische Relevanz:** Das Verbleiben von *mecA* in MRSP Bakterinen weist auf die Möglichkeit einer Verbreitung dieses wichtigen Resistenzmediators durch Bakterin-Therapie hin. Solange die Fähigkeit von Hautbakterien, nackte DNA aufzunehmen, unklar ist, sollten autogene Bakterine nur aus *mecA*-negativen *S. pseudintermedius* hergestellt werden, um das Risiko einer Verbreitung von *mecA* zu vermeiden.

## Introduction

There is an urgent need for good antimicrobial stewardship in small animal practice due to the often-close contact between pets and humans and the potential for transmission of multidrug-resistant bacteria.

Staphylococcal skin infections are amongst the most common reasons for antimicrobial prescriptions in dogs, mostly by the systemic route (1). But even when therapy has been effective, pyoderma often recurs due to e.g. underlying chronic disease which consequently leads to a need for repeated treatment. In addition, management is nowadays complicated by meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) which shares genetic, epidemiological and clinical features with the human healthcare-associated pathogen MRSA (2,3).

Autogenous *S. pseudintermedius* bacterins are gaining attention as a management option for dogs with recurrent bacterial skin infections (pyoderma) as evidence suggests that bacterins may help to prevent recurrence of infections and thus reduce the need for repeated antimicrobial prescribing (4,5).

Bacterins are formulated using phenol and formal (typically 40% saturation of formaldehyde gas dissolved in water as 100% formalin and diluted in saline to 10% formal saline) to render bacteria non-viable prior to subcutaneous injection. With bacterial cell wall antigens likely preserved, immunological mechanisms are thought to be responsible for the clinical effects, but these remain poorly understood (6).

However, the emergence of MRSP has raised concern over potential dispersal of *mecA*, the marker gene for broad β-lactam resistance in meticillin-resistant staphylococci. Since a variable effect of formalin on DNA has been reported (7), subcutaneous bacterin injections made from MRSP isolates may introduce resistance gene material into the skin with opportunity for uptake by resident bacteria through competence and transformation (8).

This study investigated the presence and integrity of *mecA* after phenol and formalin processing during the formulation of autogenous bacterins from MRSP.

## Methods

Twenty staphylococcal isolates from canine pyoderma (12 MRSP [9,10], 7 MRSA [11], and one meticillin-susceptible *S. pseudintermedius*, MSSP [10]) were included and all were

prepared in duplicate. For all isolates, species (thermonuclease gene) and methicillin-resistance had been confirmed by PCR as part of previous studies.

For bacterin formulation a previously-described method was followed (4), with the exception of a final steam autoclaving step. Pellets from bacterial overnight growth in 10 ml tryptone soy broth (ThermoFisher Scientific Ltd., Oxford, U.K.) were washed three times with 20 ml of 0.5 % sterile phenol saline (2.5 g phenol, VWR International Ltd., Lutterworth, U.K.), vortexing, centrifuging at 3000 rpm for 15 min and removing the supernatant between each step. Pellets were then resuspended in 10 ml phenol saline and 0.1 ml of 10 % formal saline (SDS Formal Saline 10 %, Solmedia Ltd., Shrewsbury, U.K.) was added and vortexed. In addition, growth pellets from five meticillin-resistant staphylococci (MRS: 2 MRSA, 3 MRSP) were processed using the same phenol washes or formal addition but as a single step to determine if one aspect of the method impacted primarily on DNA viability.

Sterility of bacterins and phenol- and formal-only suspensions was tested by overnight incubation on 5% sheep blood agar (ThermoFisher Scientific Ltd.) at 37 $^{o}$C.

DNA was extracted from all bacterins (after addition of 3 µl of 5 mg/ml lysostaphin (Merck Life Science UK Ltd., Gillingham, U.K.) to improve cell lysis). Firstly, using a standard bacterial genomic extraction kit (EdgeBio PureElute Bacterial Genomic Kit, EdgeBio, San Jose, CA, U.S.A.); secondly, one recommended for formalin-fixed tissue samples which reverses formalin-related crosslinking by methylene bridges between nucleic acids and proteins (Qiamp DNA FFPE Tissue Kit, Qiagen). For the five isolates processed with phenol or formal alone, only the standard extraction was used.

The presence of *mecA* was investigated after PCR (all 20 bacterin preparations and 5 MRS after phenol and formal alone) of a 310 bp amplicon (12). To examine the integrity of *mecA* after bacterin processing, the amplicon from each of five randomly chosen MRS was sequenced and aligned to a known segment within SCC*mecA* I of MRSA COL (Accession number NC_002951.2) (13). Post-*mecA*-PCR amplicons were cleaned (Monarch PCR and DNA clean up kit 5ug; New England Biolabs Inc., Hitchin, U.K.), quantified (Nanodrop ND-1000; ThermoFisher Scientific), aliquots diluted to approximately 20 ng/µL in TE buffer and submitted with PCR primers (5 mM) for Sanger sequencing (Source BioScience, Cambridge, U.K.). Sequences were examined using BioEdit Sequence Alignment Editor v.7.2.5 (14) to ensure that all bases were correctly assigned during automatic sequence reading. Forward and reverse sequences were aligned using NCBI Blast (15) to identify mismatches between the strands. Mismatches were referred to the original sequence trace to confirm that this was due to a mis-read rather than a true discrepancy between strands. On confirmation, forward-aligned sequences were compared using EMDL-EBI Clustal Omega Multiple Sequence Alignment Tool (16) to the COL MRSA genome.

## Results

All suspensions were sterile following bacterin processing while growth was seen on blood agar from four of twenty preparations (including duplicates) after phenol or formal treatment alone.

The target amplicon of *mecA* was present in all 5/5 isolates after phenol or formal only processing following FFPE DNA extraction; after full bacterin processing, *mecA* was seen in all 19/19 meticillin-resistant isolates after FFPE extraction (Figure 1a) and in 16/19 isolates after standard extraction (Figure 1b). Findings for duplicates were identical for all and no bands for *mecA* were seen in any of the gels for the MSSP isolate (ED99). The amplicons from all five isolates aligned with 100 % homology to COL MRSA, indicating no degradation of this portion of the *mecA* gene during bacterin processing.

## Discussion

These results confirm that even though bacteria were non-viable after phenol and formalin processing, the commonly screened for *mecA* amplicon of a critical multidrug-resistance gene in staphylococci remained unharmed. The lack of complete DNA destruction after formalin fixation processing is not new and is already allowing modern molecular analyses to answer critically important research questions from historical archived specimens (17). What is important though is that the identification of *mecA* in autogenous bacterins calls for caution when considering autogenous bacterins in the management of recurrent MRSP infections.

One caveat of our work was that only a 310 bp amplicon of the approximately 2 kbp sized *mecA* was multiplied and analysed. Breaks in the DNA may have occurred on either side of this amplicon. In that case though, different size bands would have been expected after PCR which was not the case with any of the isolates; furthermore, this particular amplicon has been shown to be highly conserved within the different SCC*mec* variants of *S. aureus* and *S. pseudintermedius* that have been described (18,19).

Whether the persistence of *mecA* translates into a real risk *in vivo* if injected into subcutaneous tissue, depends on several factors, in addition to antimicrobial selection pressure. Potential pathogens need to be present at subcutaneous injection sites which has previously been shown to be the case in human skin (20) but no bacterial DNA was recently found in normal subcutis and dermis of seven dogs (21). Bacteria then need to be able to take up external naked DNA which many bacteria can do through transformation, at least in vitro, although some are also naturally competent, particularly if culture density is high (8). The transfer of *mecA* from *S. sciuri* into *S. aureus* in the evolution of MRSA is the best current working example of clinically relevant transmission of DNA between different staphylococcal species (22). However, in addition to endogenous mammalian DNAses able to degrade DNA in tissue (23), *S. aureus* and likely other staphylococcal species can also protect themselves against foreign DNA by various restriction modification enzymes (RM systems) and CRISPR systems making acquisition of nucleic acid material a relatively rare event (24).

## Conclusion

On balance, while the chances of functional *mecA* uptake by skin bacteria are likely minimal, uptake cannot be excluded. However, MRSP pyoderma, at least superficial pyoderma for which bacterin therapy would typically be recommended, can be resolved in most dogs using topical antimicrobial therapy (3), and Staphage Lysate (SPL, Delmont

Laboratories, Swarthmore, PA, U.S.A.), a phage lysate of well-characterised *S. aureus* cultures, may be also be considered for therapy (25). It would therefore be prudent to resolve an MRSP pyoderma first and only consider autogenous *S. pseudintermedius* bacterins once *mecA*-negative *S. pseudintermedius* can be isolated from recurrent infections again. Repeat sampling over several months may be required in some dogs.

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## Figures

Figure 1: Agarose gel electrophoresis (2% agarose) of PCR amplified products showing presence of *mecA* after bacterin processing of staphylococcal isolates, following DNA extraction using a) kit for formalin-fixed tissue (FFPE), or b) standard bacterial DNA extraction kit.
Lane M: 100bp DNA ladder; lanes 1-7: meticillin-resistant *Staphylococcus aureus* (MRSA); lane 8: meticillin-susceptible *S. pseudintermedius* (MSSP); lanes 9-20: meticillin-resistant *S. pseudintermedius* (MRSP); +: positive control MRSA DNA; -: negative control MSSP DNA; B: blank PCR reaction without DNA. Source: © S. Frosini

Abbildung 1: Agarose-Gel-Elektrophorese (2% Agarose): *mecA* Banden aus Staphylokokken Bakterinen nach PCR Amplifizierung. DNA Proben extrahiert mit a) FFPE-Extraktions-Kit für formalinfixierte, paraffineingebettete Proben, oder b) einer Standard DNA Extraktions Methode.
Spur M: 100bp DNA Leiter; Spuren 1-7: metizillin-resistenter *Staphylococcus aureus* (MRSA); Spur 8: metizillin-empfindlicher *S. pseudintermedius* (MSSP); Spuren 9-20: metizillin-resistenter *S. pseudintermedius* (MRSP); +: positive Kontrolle (MRSA DNA); -: negative Kontrolle (MSSP DNA); B: Kontroll Spur nach PCR ohne DNA. Quelle: © S. Frosini