Acta vet. scand. 1980, *21*, 305—311.

From the National Veterinary Institute, Oslo, and the Department of Animal Genetics and Breeding, Agricultural University of Norway, Ås-NLH, Norway.

ANTIPENICILLINASE IN THE SERUM OF DAIRY COWS

By

Ø. Lie and *H. Solbu*

LIE, Ø. and H. SOLBU: *Antipenicillinase in the serum of dairy cows.* Acta vet. scand. 1980, *21*, 305—311. — Serum from dairy cows was tested for inhibitory effect on penicillinase from a penicillin-resistant Staphylococcus aureus strain of mastitic origin. Among cows with subclinical mastitis caused by penicillin-resistant S. aureus there was a significantly higher frequency of individuals with penicillinase-inhibiting serum than among healthy cows. Among the subclinical cases, a foregoing penicillin treatment of clinical mastitis appeared to increase the serum antipenicillinase activity.

serum antipenicillinase; penicillin resistance; Staphylococcus aureus; bovine mastitis.

Penicillins, cephalosporins and other β-lactam antibiotics have the β-lactam bond in their chemical structure. These chemotherapeutics are therefore exposed to the action of the β-lactamase enzymes. The β-lactamases are commonly referred to as penicillinases and are responsible for the major part of the resistance of Staphylococcus aureus to penicillins (*Workman & Farrar* 1970, *Richmond* 1977). The penicillinases from Gram-positive bacteria have been subjected to fairly intensive immunochemical study, one of the conclusions being that there are few or no cross-reactions between enzymes from different species (*Pollock* 1964).

Interactions of penicillinases with their antisera have also been thoroughly studied. *Housewright & Henry* (1947) demonstrated the complete inactivation of penicillinase after incubation with rabbit antipenicillinase serum for 1 h at 37°C, using penicillinsodium as substrate. They found increased sensitivity of organisms to penicillin in the presence of antipenicillinase serum,

but only as regards those organisms which produced extracellular penicillinase (S. aureus and Bacillus cereus). It was later reported that the effect of these immune sera seems to vary markedly, the stimulating effect included. Among other factors, this variation probably depends on the strain of exopenicillinase (bacterium) and the type of substrate (antibiotics) used (*Pollock*). So far, no investigations seem to have been carried out on antipenicillinase in "normal" sera of animals with penicillinase-producing microbes in their organs. It therefore seems worth-while to perform such studies and if possible to estimate the practical value of "normal serum antipenicillinases" if they do exist.

The purpose of the present investigation was to find out if sera from dairy cows possess any detectable antipenicillinase activity. Furthermore, the intention was to study if any such activity is influenced by udder infection with penicillin-resistant S. aureus or by penicillin treatment of clinical mastitis.

MATERIALS AND METHODS

Source of material

From computer-stored data, 43 cows with subclinical mastitis due to penicillin-resistant S. aureus and 36 healthy cows were selected. The latter were to serve as control animals. Blood samples had been collected from all cows at the time of the milk sampling. Furthermore, the clinical histories of the subclinical cases for the 4-month period immediately prior to sampling, were available from computer-stored "health card" data. These are field data from the disease recording scheme for dairy cattle in Norway. A detailed description of the practical operation of this scheme has been given by *Solbu* (1978).

Production of penicillinase

A penicillin-resistant S. aureus strain* of mastitic origin was allowed to grow for about 72 h at 37°C on a nutrient broth, containing 100 i.u. of benzylpenicillinsodium per ml. The complete degradation of penicillin was determined by the lack of growth inhibition effect on the penicillin sensitive organism Bacillus stearothermophilus var. calidolactis**. The only purification

* No. 3380, Culture collection of the Department of Microbiology and Immunology, Veterinary College of Norway, Oslo.

** Art. no. 10648, E. Merck, Darmstadt, W. Germany.

procedure performed was the sterile filtration of the broth through a filter (Millipore) with a pore size of 0.22 μm.

Antipenicillinase assay

The serum antipenicillinase activity was measured using an inhibition method. The serum sample and the penicillinase were mixed in equal amounts and incubated for 1 h at 37°C in a small tube. The preincubated serum/penicillinase solution and benzylpenicillinsodium (1 i.u. per ml) were then mixed in a proportion of 2 : 1 and incubated for another hour at 37°C. At the same time, equal volumes of the penicillinase and benzylpenicillin were incubated in 1 tube and penicillin in another as controls. 15 μl of the benzylpenicillin solution was deposited into a well (A) in an agar plate seeded with B. stearothermophilus var. calidolactis and 30 μl of the penicillin/penicillinase solution deposited into a second well (B). As a control for possible growth inhibition effect of the serum on the bacterium, 15 μl of the serum sample was added to a third well (C). Finally 45 μl of the serum/penicillinase /penicillin solution was placed in a fourth well (D). The plates (1 plate for each of the 79 samples) were kept at room temperature for 1 h, allowing the admixtures to diffuse into the agar, and then incubated for 3 h at 60°C. A positive antipenicillinase effect of the serum was expected to appear as a growth inhibition zone around well D.

RESULTS

A clear penicillinase-inhibiting effect of serum (well D) is shown in Fig. 1. The serum had not completely inactivated the penicillinase as can be seen by comparing the larger inhibition zone around well A, containing only penicillin. However, compared with well B, containing penicillin/penicillinase, and with well C, containing only serum, the inhibition zone around well D demonstrates the significant antipenicillinase activity of this serum sample.

As seen in Table 1, serum from 34 of the 79 cows showed penicillinase-inhibiting activity. As many as 8 of the 36 control animals also showed antipenicillinase activity. However, the number of animals with positive serum was significantly higher in the test group than in the control group ($P < 0.001$, χ^2-test).

The clinical histories of the cows, which were retrieved by means of the "health card" system, are given in Table 2. Fifteen

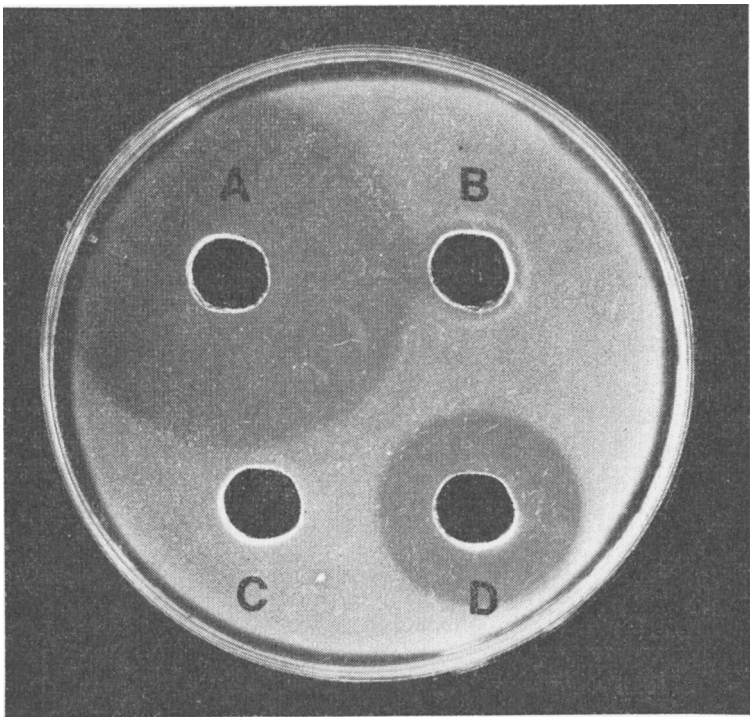

Figure 1. Inhibition test indicating the antipenicillinase activity of serum from a dairy cow (well D) on an agar plate, fitted with 4 wells, seeded with B. stearothermophilus var. calidolactis.

Admixtures in the wells:

A: Benzylpenicillinsodium.
B: Penicillin/penicillinase, preincubated for 1 h at 37°C.
C: Undiluted serum.
D: Two-steps preincubated serum/penicillinase/penicillin.

The volumes were adjusted so that wells A, B and D contained equal amounts of penicillin, B and D equal amounts of penicillinase and wells C and D equal volumes of serum. The plate was incubated for 3 h at 60°C.

cows with prior clinical mastitis were recorded among the 43 animals which had subclinical infections at the time of sampling. The frequency of previously treated cases of mastitis was higher among the cows with penicillinase-inhibiting serum than among those with negative serum. The difference was close to significance at the level of 0.05 ($\chi^2 = 3.60$, d.f. $= 1$).

Table 1. The incidence of cows with penicillinase-inhibiting serum among 79 dairy cows grouped according to udder health status.

	Cows with penicillinase-inhibiting serum	Cows with non-inhibiting serum
Cows with subclinical mastitis caused by penicillin-resistant S. aureus	26	17
Healthy cows	8	28

Table 2. The incidence of previously treated cases of mastitis among cows with penicillinase-inhibiting serum and among cows with negative serum.

	Cows previously treated for clinical mastitis	Cows not previously treated for clinical mastitis
Cows with penicillinase-inhibiting serum	12	14
Cows with non-inhibiting serum	3	14

DISCUSSION

There are reasons to believe that the inhibition test performed in this experiment is rather specific. As the test is based on penicillinase activity and the inhibtion of this activity by serum, it might be assumed that the serum samples from the positive cows contained antibodies against penicillinase. Moreover, if the recorded inhibition is solely due to specific antibodies, these must represent so-called anti-enzymes since they interfere with the activity of an enzyme. Comprehensive serological studies have been carried out on antienzymes in sera and secretions of animals against other bacterial enzymes, including proteinases (*Fossum* 1971) and deoxyribonucleases (*Sandvik* 1974, 1975, *Gudding* 1977).

The antipenicillinase effect demonstrated in this experiment might be due to unspecific inhibitors of a non-antibody nature. However, the fact that there was a significantly higher incidence of animals with inhibiting serum among the cows with the penicillinase-producing S. aureus in their udders than among healthy ones (Table 1) does not support such an objection. It is therefore

reasonable to suggest that the difference between the test and control group was caused by a difference in specific antibodies rather than by unspecific inhibitors against penicillinase.

Another observation supporting this suggestion is the fact that the incidence of prior clinical mastitis was almost significantly higher among the cows with inhibiting serum than among cows with non-inhibiting serum (Table 2). This presumably means that the cows with serum antipenicillinase were the ones treated most frequently with penicillin prior to sampling. It should be emphasized that the presence of penicillin is probably necessary to induce the production of penicillinase by S. aureus in an amount sufficient to stimulate a detectable antibody response in the cow. In the absence of enzyme induction, the penicillinase will mainly remain as a precursor inside the bacterial cell, thus being unavailable as an immunogen (*Pollock* 1964).

In the present study the antipenicillinase activity has been recorded as an "all or non" trait. However, the described inhibition test revealed considerable variation among animals, expressed through the diameter of the inhibition zone. The method might therefore be usable for quantitative determination of antipenicillinase. Moreover, by combining electrophoresis of the serum with this inhibition test, the activity of the antienzyme antibodies might be distinguished from activity due to possible unspecific inhibitors.

The present findings suggest that "normal" serum from cows may contain detectable antipenicillinase activity, probably due to immunogenic stimulation by the penicillin-induced penicillinases produced by resistant S. aureus during penicillin treatment. These findings may have several implications. Firstly, antipenicillinase in routine laboratory samples of serum or milk might be a useful indicator of herds with penicillin-resistant microbes. Secondly, it might be possible to use penicillinase in the manufacture of bovine vaccines to stimulate the production of antibodies against penicillinase from penicillin-resistant organisms. Thirdly, antipenicillinase serum might be applied therapeutically together with penicillin in order to protect the drug from enzymatic inactivation. Finally, the genetically influenced ability to produce antibodies to S. aureus penicillinase in cattle could be tested, using the procedure applied in testing the antibody response to other immunogens (*Lie* 1979). This procedure could perhaps be included in general selection pro-

grammes to produce cows genetically "resistant" to penicillin-resistant S. aureus.

REFERENCES

Fossum, K.: Proteolytic enzymes and biological inhibitors. VI. Antibodies in animal sera against the proteinase of Corynebacterium pyogenes. Acta path. microbiol. scand. 1971, *79*, 117—122.

Gudding, R.: An agar diffusion method for the determination of antibodies against Staphylococcus aureus deoxyribonuclease. Acta vet. scand. 1977, *18*, 480—493.

Housewright, R. D. & R. J. Henry: Studies on penicillinase. III. The effect of antipenicillinase on penicillin resistant organisms. J. Bact. 1947, *53*, 241—247.

Lie, Ø.: Genetic analysis of some immunological traits in young bulls. Acta vet. scand. 1979, *20*, 372—386.

Pollock, M. R.: Stimulating and inhibiting antibodies for bacterial penicillinase. Immunology 1964, 7, 707—723.

Richmond, M. H.: Enzyme/antiserum interactions of β-lactamases. *In* Immunochemistry of Enzymes and their Antibodies. M. R. J. Salton (ed.). John Wiley & Sons, New York 1977.

Sandvik, O.: The occurrence of antibodies against staphylococcal deoxyribonucleases in blood sera from different species. Acta vet. scand. 1974, *15*, 631—635.

Sandvik, O.: The occurrence of antibodies against staphylococcal deoxyribonucleases in bovine milk. Acta vet. scand. 1975, *16*, 140—142.

Solbu, H.: Breeding for improved disease resistance, with special emphasis on a practical method of collecting data. 1978. EAAP, 29th Ann. Meeting, Stockholm, C/1.03, 8 pp.

Workman, R. G. & W. E. Farrar jr.: Activity of penicillinase in Staphylococcus aureus as studied by the iodometric method. J. infect. Dis. 1970, *121*, 433—437.

SAMMENDRAG

Antipenicillinase i serum hos kyr.

Serum fra mjølkekyr ble testet for inhiberende effekt overfor penicillinase fra en penicillin-resistent Staphylococcus aureus-stamme isolert fra bovin mastitt. Blant kyr med subklinisk mastitt forårsaket av penicillin-resistent S. aureus var det en signifikant høyere frekvens av individer med penicillinase-inhiberende serum enn blant friske kyr. Blant de subkliniske tilfellene syntes en forutgående behandling for klinisk mastitt å øke serum-antipenicillinaseaktiviteten.

(Received March 3, 1980).

Reprints may be requested from: Øystein Lie, the National Veterinary Institute, P. O. Box 8156 Dep., Oslo 1, Norway.