

Production of penicillin-specific polyclonal antibodies for a group-specific screening ELISA

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(Received 2 March 2007; accepted 12 November 2007)

Abstract

Polyclonal penicillin-specific antibodies were obtained after the immunization of three rabbits with physiological penicillin-protein conjugates. The broad-specificity of the antisera was improved by alternate immunizations using immunogens with different penicillins as haptens. For the three antisera, the detection of ampicillin, amoxicillin, benzylpenicillin, oxacillin, cloxacillin and dicloxacillin was more sensitive in a competitive ELISA coated with the antibodies (antibody cELISA) compared to a competitive ELISA coated with a penicillin-protein conjugate (antigen cELISA). The detection of all penicillins in buffer solutions at concentrations below the MRL was achieved in the antibody cELISA when the penicillins were hydrolyzed with Penicillinase I. A simple extraction procedure was developed for the detection of penicillins in incurred porcine tissues using the antibody cELISA. Tissue fluids were used instead of the whole meat matrix, which simplified the sample preparation. Before analysis, the fluids were treated with kaolin to reduce the background signals in ELISA. The specificity, sensitivity, repeatability, decision limit ($CC\alpha$) and detection capability ($CC\beta$) were determined. The suitability of the antibody cELISA and the respective extraction procedure for screening purposes was demonstrated by comparison of the analysis of incurred samples using different screening assays. Almost 100% correlation ($r=0.96$) was found between the ELISA and a commercial immunochemical method, the ParallaxTM assay.

Keywords: *Penicillin, polyclonal antibodies, ELISA, extraction.*

Introduction

Penicillins are frequently used in veterinary medicine to treat and prevent bacterial infections. They are also used as feeder additive to increase feed efficiency, promote growth and prevent disease. Consequently, residues can be found in food derived from treated animals and are detrimental to the health of consumers (Milhaud & Person 1981; Boison 1995). Immunological assays are commonly used to screen food

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products for the presence of anti-microbial drug residues (Allison 1985; Charm & Chi 1988; Kanavanagh 1989).

For the development of a penicillin-specific immunological assay, monoclonal or polyclonal antibodies (pAbs) against the common penicillin structure are required. Monoclonal antibodies bind to one epitope of a molecule, and are, therefore, very specific, have a high degree of purity, are easily reproducible, and are thus very suitable for the development and standardization of a test system. In contrast, large variations can occur during the production of pAbs. On the other hand, polyclonals are less influenced by physiological parameters, such as pH and temperature, and have a higher avidity compared to monoclonals (Tijssen 1985; Booman 1988; Cambell 1994). In ELISA, antibodies with high affinity or avidity are required because low affinity interactions are rapidly dissociated during the wash steps. As a result, it is possible that an ELISA using pAbs is more sensitive compared to an ELISA using monoclonals (Tijssen 1985).

The production of a sensitive penicillin-specific antiserum is difficult. Different chemical methods have been reported to couple ampicillin to the carrier protein via the amino group to synthesize immunogens, which are able to induce antibodies against the intact penicillin structure (Kitagawa et al. 1978; Nagakura et al. 1991; Usleber et al. 1998; Strasser et al. 2003). Usleber et al. (1998) obtained rabbit antiserum for which an ELISA, able to detect several penicillins at concentrations in the range of 5–20 ng/ml, could be developed. Strasser et al. (2003) have reported a cELISA allowing the detection of penicillins in milk in a range of 1 ng/ml (for penicillin G) to 6 ng/ml (for dicloxacillin). Using 6-aminopenicillanic acid as hapten for the development of a penicillin-protein conjugate with intact β -lactam structure provided weak immunogens (De Leuw et al. 1997). Other attempts to produce group-specific antisera using the immunogen β -lactam coupled to the carrier protein through the carboxy-group led to highly specific antisera, but again was not sensitive enough (Kachab et al. 1992; Usleber et al. 1994). Direct covalent binding of the penicillin to the carrier protein (physiological conjugation) led to antisera highly reactive for the open β -lactam ring structure and less against the native molecule (Rohner et al. 1985; Grubelnik et al. 2001).

The aim of our research was to develop an ELISA enabling the detection of penicillin in porcine tissues. Therefore, monoclonal and polyclonal rabbit antibodies against the common penicillin structure were produced using physiological penicillin-carrier protein conjugates as immunogens. The production of these conjugates and monoclonal antibodies has already been described (Cliquet et al. 2001). To improve the broad-specificity of the rabbit antisera, immunogens with different penicillins were used for subsequent immunization. The antisera were analyzed in an antigen and in an antibody competitive (c)ELISA for detection of different penicillins.

An appropriate extraction procedure was developed for the detection of penicillins in porcine tissues. An investigation if the ELISA could be validated according to the Commission Decision 2002/657/EC was undertaken (Anonymous 2002). Some of the relevant performance characteristics for the validation of both assays were determined and are discussed in this study. Finally, the analysis of incurred samples using the ELISA procedures was compared to the analysis of the same samples using other screening assays.

Material and methods

Reagents and chemicals

Benzylpenicillin, oxacillin, dicloxacillin, cefaclor, cephadrin, cephalixin, sulphanilamide, chloramphenicol, bovine serum albumin (BSA), kaolin (hydrated aluminium silicate), Penicillinase (EC 3.5.2.6, type 1, from *B. cereus*) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma Chemicals (Bornem, Belgium). The TMB enzyme-substrate solution was prepared by adding 100 µl of a TMB stock solution (10 mg TMB dissolved in 1 ml dimethylsulphoxide) to 10 ml phosphate-citrate buffer pH 5 (25.7 ml 0.2 M Na₂HPO₄ + 24.3 ml 0.1 M citrate + 50 ml distilled water) supplemented with 1.3 µl H₂O₂. Amoxicillin and cloxacillin were obtained from ICN Biochemicals (Asse-Relegem, Belgium), and ampicillin and ABTS (2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (Tablet and buffer) were from Roche Diagnostics (Brussels, Belgium). Tween-20 (polyoxyethylene sorbitan monolaurate) was purchased from Merck-Belgolabo (Overijse, Belgium). Peroxidase-labeled swine anti-rabbit immunoglobulins (code No. P0217) were from DAKO Diagnostica (Prosan, Ghent, Belgium). EZ-linkTM Sulfo-NHS-LC-Biotin was purchased from Pierce (Perbio, Erembodegem-Aalst, Belgium). Complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were provided by Difco Laboratories, Biotrading (Bierbeek, Belgium). ELISA Microtitre plates (Maxisorp[®]) were provided by NUNC (VWR International, Leuven, Belgium). All other chemicals were of reagent grade.

The ELISA coating antigen, an ampicillin-ovalbumin conjugate (ampMBSova), was synthesized with an activated ester method using the hetero-bifunctional reagent *m*-maleimido benzoyl-*N*-hydroxisuccinimide ester (MBS) (Cliquet et al. 2001).

Production of pAbs

New Zealand white rabbits were immunized subcutaneously with 500 µg of the physiological penicillin-carrier conjugate (Cliquet et al. 2001). For the first immunization, the conjugate was emulsified in sterile saline (500 µl) and CFA (500 µl). For all subsequent immunizations, at six-week intervals, IFA was used. Blood was sampled two weeks after each immunization. The serum was collected (3000 g, 20 min) and treated with kaolin (Van den Broeck et al. 1999). Therefore, one part of serum was mixed with four parts of kaolin and incubated for 30 min at room temperature. The mixture was centrifuged (3000 × *g*, 20 min) and the supernatant was stored frozen. The pAbs were analyzed in cELISAs.

Biotinylation of penicillin

Ampicillin or amoxicillin (1.2 mg) were diluted in 300 µl phosphate buffer 0.1 M, pH 7.2 and added to 3.7 mg of a succinimide ester-biotin conjugate (EZ-linkTM Sulpho-NHS-LC-Biotin) diluted in 700 µl phosphate buffer 0.1 M, pH 7.2. The reaction mixture was incubated for 24 h at room temperature while shaking. It was not necessary to remove the unreacted biotin: the conjugate was diluted at least 1000 times in ELISA. At this dilution, no background signals were observed. Aliquots of the conjugate were stored at -20°C.

Competitive ELISA coated with antigen (antigen cELISA)

Microtitre plates were coated overnight with 2.5 µg/ml ampMBSova (Cliquet et al. 2001) diluted in bicarbonate coating buffer (0.05 M; pH 9.4) followed by blocking for 2 h at 37°C with 5% glycine in coating buffer. Subsequently, penicillin was added (100 µl/well) diluted in PBS (0.15 M; pH 7.4) containing 3% BSA and 0.05% Tween-20 (dilution buffer) together with an appropriate concentration of the penicillin-specific pAbs: pAb K2, pAb K6 or pAb K8 (100 µl/well). The microtitre plates were then incubated for 1 h at 37°C. Next, the plates were incubated with peroxidase-labeled swine anti-rabbit antibodies in a dilution buffer for 60 min at 37°C. Between each step, the plates were washed three times with PBS containing 0.05% Tween-20. Finally, the plates were incubated for 1 h at 37°C with the enzyme substrate solution (ABTS, 50 µl/well). The color development (absorbance (A) or optical density (OD)) was measured at 405 nm using an ELISA reader (Spectrofluor, TECAN).

Competitive ELISA coated with antibody (antibody cELISA)

Microtitre plates were coated overnight with pAb K2, pAb K6 or pAb K8 (1/1000) diluted in bicarbonate coating buffer (0.05 M; pH 9.4). After coating, the plates were blocked for 2 h at 37°C with 0.2% Tween-80 in coating buffer. Subsequently, penicillin was added in dilution buffer, as for the antigen cELISA. The plates were then incubated for 1 h at 4°C. At this low temperature, Penicillinase I will be inactivated, and thus the test penicillin will no longer hydrolyze. Next, 100 µl of biotinylated ampicillin (for pAb K2 and pAb K6) or biotinylated amoxicillin (for pAb K8) in dilution buffer was added, without washing the plate, for 30 min at 4°C. The other wash steps occurred as for the antigen cELISA. Then, the plates were incubated for 30 min at 37°C with streptavidin-peroxidase conjugate in dilution buffer. Finally, the enzyme substrate solution (TMB) was added to the plates for 1 h at 37°C. The color development was measured at 650 nm.

Interpretation of the ELISA results

The competition between free penicillin in the sample and the biotinylated penicillin or coated penicillin was calculated with the formula:

$$\text{competition (\%)} = (1 - (B/B_o)) * 100$$

where B is the absorbance of a tested sample solution, and B_o is the absorbance of a similar solution without free penicillin.

The calibration curve was obtained by plotting the concentration of a standard dilution of penicillin against the absorbance measured for the binding of the antibodies (in the antigen cELISA) or biotinylated penicillin (in the antibody cELISA) in the presence of standard dilutions.

The limit of detection (LOD = average signal $-3 \times \text{SD}$) and IC₅₀-value (concentration of free penicillin for which the binding of the antibodies is inhibited for 50%) in ng/ml is obtained by extrapolation of the response or absorbance in a calibration curve.

Hydrolysis of the sample penicillin

Without hydrolysis. Penicillin was diluted in PBS just before use to avoid spontaneous hydrolysis.

Spontaneous hydrolysis. Penicillin was diluted in PBS and incubated overnight at room temperature.

Enzyme mediated hydrolysis. Penicillinase I was diluted in PBS (1000 unit/ml) and stored at -80°C . Prior to use, the Penicillinase I stock was further diluted (0.1 U/ml). Five microlitres of this Penicillinase I dilution was added to 1 ml sample. Next, the mixture was incubated for 1 h at 37°C followed by 30 min incubation at 4°C to inactivate the enzyme (Medina et al. 1998).

Repeatability of the ELISA

Samples of amoxicillin in buffer solution (eight different concentrations) were analyzed in the antibody cELISA six times in the same assay (intra-assay variation) and in six different assays (inter-assay variation). The analyses were repeated on six different days (inter-day variation).

Origin of the animal tissue samples

The porcine kidneys used as reference control samples or for the determination of $\text{CC}\alpha$ and $\text{CC}\beta$ were obtained at slaughter from animals reared on an experimental unit without penicillin treatment.

The samples (15 porcine or bovine kidneys) analyzed in the antibody cELISA, the ParallaxTM assay and the NBKT were collected at slaughterhouses by the Federal Agency for the Safety of the Food Chain and were stored at -20°C until analysis.

Sample analysis in the antibody cELISA

Sample preparation. Tissue fluids were collected after centrifugation of minced tissue (10 g; 10 min, $2000 \times g$). Kaolin (800 μl of 25% kaolin in PBS) was added to 100 μl tissue fluid. The mixtures were shaken thoroughly and incubated for 30 min at room temperature. After centrifugation ($12\,000 \times g$, 10 min), 650 μl of the supernatant was added to 350 μl PBS supplemented with BSA (6%) and Tween-20 (0.1%).

Decision limit and detection capability. Fourteen control porcine kidneys samples spiked with amoxicillin at the MRL (50 ppb) were analyzed on different days in the antibody cELISA. The average B/B_0 for $\text{CC}\alpha$ and $\text{CC}\beta$ was calculated according to the Commission Decision 2002/657/EC. The corresponding concentrations were obtained by plotting the competition values ($= (1 - B/B_0) * 100$) in a calibration curve of amoxicillin in buffer solution.

Incurred sample analysis. Test penicillins of the sample extracts were hydrolyzed with Penicillinase I. Fluids from negative tissues and fluids from negative tissues spiked with amoxicillin at the MRL-value (50 ppb) were analyzed as negative and positive control, respectively. A serial dilution of amoxicillin in buffer solution or in the

extraction fluid of the corresponding control tissue was used to construct a calibration curve (concentration vs. competition). The sample analyses were considered valid if the competition value for the positive control sample was in the range of the expected value ($67 \pm 4\%$). A sample was considered non-compliant when the concentration was higher than the detection capability ($CC\beta$), compliant when lower than the decision limit ($CC\alpha$), and suspected when between $CC\alpha$ and $CC\beta$.

The new Belgian kidney test (NBKT)

Paper disks (12.7 mm diameter) impregnated with kidney fluid and a piece of kidney cortex (a few mm) were incubated on an agar layer (pH 7.2) previously seeded with a susceptible bacterial strain (*Bacillus subtilis*). The growth inhibition zone was measured in millimeters. The samples were analyzed twice. Samples producing an inhibition zone >20 mm for the paper disk (zone inclusive the diameter of the disk), and >2 mm around the cortex were considered positive for the presence of antimicrobial substances.

The ParalluxTM assay (IDEXX Laboratories)

The Parallux assay was developed for the detection of ampicillin, amoxicillin and benzylpenicillin in milk (Huth et al. 2002), but it can also be used for the detection of these penicillins at the MRL in bovine and porcine kidney tissues (Okerman et al. 2003). The analyses of incurred samples were performed as previously described by Okerman et al. A test sample was considered non-compliant if the signal obtained for the kidney sample was higher than 1.50 (Okerman et al. 2003).

Results and discussion

Development of penicillin-specific antibodies and an antigen cELISA

The polyclonal antisera were obtained after the immunization of three rabbits (K2, K6, K8) with physiological ampicillin- and benzylpenicillin-protein conjugates (pAb K2) or with physiological ampicillin-, benzylpenicillin-, oxacillin and dicloxacillin-protein conjugates (pAb K6 and pAb K8).

Based on the allergenicity of penicillins, no differences in immunogenicity should be observed when using different penicillins as haptens. The β -lactam ring of all penicillins spontaneously opens under physiological conditions, forming the penicilloyl group. This group is designated as the major determinant because approximately 95% of the penicillin molecules that irreversibly combine with proteins form penicilloyl moieties. Penicillins are also degraded by other metabolic pathways to form, in smaller quantities, other antigenic derivatives, called minor determinants. Both major and minor determinants can bind to proteins and elicit an immune reaction (Chowdhury & Lieberman 1999). Thus, a strong immune response should be elicited with physiological penicillin-protein conjugates, independent of the penicillin used as hapten (Katsutani & Shionoya 1993). This was in accordance with our results, since high serum antibody titers were obtained after immunization of rabbits and mice (Cliquet et al. 2001).

Patients allergic to any member of the penicillins should be considered allergic to all of them (Raynor 1997). Thus, cross-reactivity for the other members of the penicillin

Table I. IC₅₀-values (ng/ml) for six penicillins in buffer not hydrolyzed and analyzed in the antigen cELISA with polyclonal antibodies pAb K2, pAb K6 and pAb K8.

Polyclonal	Hydrolysis	IC ₅₀ -values (ng/ml) for penicillins ^a in the antigen cELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	No	1,000	6,000	1,800	10,000	8,000	20,000
pAb K8	No	400	4,000	3,000	10,000	10,000	30,000
pAb K6	No	200	30,000	9,000	15,000	8,000	8,000

^aamp, ampicillin; amox, amoxicillin; bpg, benzylpenicillin; ox, oxacillin; clox, cloxacillin; diclox, dicloxacillin.

family was expected and obtained. The IC₅₀-values of the three antisera for six penicillins analyzed in the antigen cELISA are shown in Table I. Although four different conjugates were used for the immunization of K6 and K8 in contrast to only two different immunogens for K2, the same pattern of recognition was obtained for pAb K2 and pAb K8, and a slightly different pattern for pAb K6 (Table I). Ampicillin and benzylpenicillin are best recognized by pAb K2 and pAb K8, and ampicillin by pAb K6. Amoxicillin is also very well recognized by pAb K2 and pAb K8, but not by pAb K6 (which recognizes amoxicillin the least). The antisera pAb K6 is more sensitive for cloxacillin and dicloxacillin compared to pAb K8, despite the fact that both antisera were obtained after immunization with oxacillin and dicloxacillin-protein conjugates. Even pAb K2 is more sensitive for those penicillins than pAb K8. From the specificity of the three antisera, it could be concluded that it is sufficient to immunize with ampicillin- and benzylpenicillin-protein conjugates for the induction of penicillin-specific antibodies, except for the dicloxacillin recognition.

However, to deduce scientifically and statistically which conjugate is the most appropriate for the development of penicillin-specific pAbs, more than three rabbits should be immunized. However, it is well known that the immune response to the same immunogen can vary between animals.

Development of an antibody cELISA

Biotin was conjugated to the side chain of ampicillin (Figure 1) and amoxicillin, leaving the common penicillin structure free for antibody binding. These conjugates allowed the development of an antibody cELISA with the three antisera. For all pAbs,

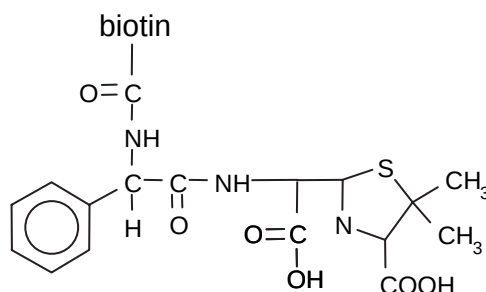


Figure 1. Biotinylated ampicillin.

Table II. IC50-values (ng/ml) for six penicillins in buffer without hydrolysis spontaneously hydrolyzed and hydrolyzed with an enzyme and analyzed in the antibody cELISA with pAb K2, pAb K6 and pAb K8.

Polyclonal	Hydrolysis	IC50-values (ng/ml) for penicillins ^a in the antibody cELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	No	55	150	450	150	800	>1,000
	Spontaneous	2	40	150	150	500	>1,000
	Enzyme	0,08	2	7	6	50	200
pAb K8	No	20	100	60	>1,000	>1,000	650
	Spontaneous	15	80	50	300	1,000	500
	Enzyme	2	3,5	0,8	10	6	10
pAb K6	No	2,5	500	250	>1,000	>1,000	>1,000
	Spontaneous	2,5	300	250	>1,000	>1,000	>1,000
	Enzyme	1,5	150	40	60	200	150

^aamp, ampicillin; amox, amoxicillin; bpg, benzylpenicillin; ox, oxacillin; clox, cloxacillin; diclox, dicloxacillin.

the detection of penicillins is more sensitive in the antibody cELISA compared to the antigen cELISA (Tables I, II, III and IV, no hydrolysis).

The difference in sensitivity between both ELISAs could be ascribed to a combination of factors, such as the use of a different competitor molecule (Choi et al. 2002), the immunogen used to produce the antibodies (Kirkley et al. 2001), as well as the set-up of the test system. In the antibody cELISA, the antibodies are immobilized and the sample is incubated in the plate for 1 h, thereafter the biotinylated penicillin is added. The penicillins in the sample can thus bind to the coated antibodies before the biotinylated penicillin does. In ELISA, dissociation rates are sometimes so low that the reactions can be considered practically irreversible. Consequently, the equilibrium may not be reached within the usual incubation time (Van Regenmortel & Azimzadeh 1994). This means that the penicillins bound to the coated antibodies will not dissociate during the assay incubation, inhibiting the binding of the biotinylated penicillin to form complexes with the coated antibodies.

In the antigen cELISA, the antibodies preferentially bind to the coated ampicillin. The binding of antibodies to the coated ampicillin is probably favored above the binding to free penicillins due to the higher affinity of the antibodies for a more constrained conformation of ampicillin. However, in the antibody cELISA, the binding of the sample penicillins is favored above the binding of the biotinylated penicillins due to the preincubation of sample and coated antibodies. As a result,

Table III. Detection limit (LOD, ng/ml) for six non-hydrolyzed penicillins in buffer analyzed in the antigen cELISA with pAb K2, pAb K6 and pAb K8.

Polyclonal	Hydrolysis	LOD (ng/ml) for penicillins ^a in the antigen cELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	No	6	200	150	500	550	1,050
pAb K8	No	20	300	60	200	500	2,000
pAb K6	No	20	2,000	300	700	200	200

^aamp, ampicillin; amox, amoxicillin; bpg, benzylpenicillin; ox, oxacillin; clox, cloxacillin; diclox, dicloxacillin.

Table IV. Detection limit (LOD, ng/ml) for six penicillins in buffer without hydrolysis, spontaneously hydrolyzed and hydrolyzed with an enzyme, and analyzed in the antibody cELISA with pAb K2, pAb K6 en pAb K8.

Polyclonal	Hydrolysis	LOD (ng/ml) for penicillins ^a in the antibody cELISA					
		amp	amox	bpg	ox	clox	diclox
pAb K2	No	1	10	30	7	60	200
	Spontaneous	0,3	3	20	7	50	200
	Enzyme	<0,1	<1	1	0,6	7	30
pAb K8	No	1,5	15	5	150	80	90
	Spontaneous	1,5	8	5	50	60	90
	Enzyme	<1	<1	<1	<1	<1	1
pAb K6	No	0,3	50	20	100	200	200
	Spontaneous	0,1	25	20	100	200	200
	Enzyme	<0,1	7	2	2	10	10

^aamp, ampicillin; amox, amoxicillin; bpg, benzylpenicillin; ox, oxacillin; clox, cloxacillin; diclox, dicloxacillin.

higher sensitivities can be measured in the antibody cELISA compared to the antigen cELISA. Preincubation of antibodies and sample penicillin or labeling of the antibodies with biotin did not improve the sensitivity of the antigen cELISA.

Specificity of the polyclonals

The pAbs-pAb K2, pAb K6 and pAb K8-are highly specific for penicillins. No cross-reactions were observed for cephradrin, cefalexin, cefazolin, clavulanic acid, sulphanilamide or chloramphenicol (50, 500 and 5000 ppb) analyzed in the antibody cELISA. Cross-reactivities were only noticed for cefaclor when analyzed at high concentration compared to the penicillins (IC₅₀ cefaclor approx. 5000 ppb compared to IC₅₀ <200 ppb for all tested penicillins; Table II). It is not clear why the antibodies recognize cefaclor, but not cefalexin or cephradrin. These three cephalosporins differ mainly in the substitution on the dihydrothiazin ring (-Cl for cefaclor vs. -CH₃ for cefalexin and cephradrin). Probably, the polyclonals mainly recognize the upper part of the thiazolidin and dihydrothiazin ring, since they do not recognize clavulanic acid (oxygen instead of sulfur). Other cephalosporins have larger side groups causing steric hindrance to the antibodies attempting to bind with the upper part of the dihydrothiazin ring.

Improving the sensitivity of the antibody cELISA by hydrolysis of the sample penicillin

Using the penicillin specific polyclonal antisera, the antigen cELISA and the antibody cELISA are still not sensitive enough to detect the penicillins at the MRL in meat samples. The MRL in meat and meat products is 50 ppb for ampicillin, benzylpenicillin and amoxicillin, and 300 ppb for oxacillin, cloxacillin and dicloxacillin (Anonymous 1990). Taking into account that the penicillin concentration is usually diluted ten times during meat sample preparation, the sensitivity of the ELISAs must be at least ten times lower than the MRL. None of the three antibody cELISAs or antigen cELISAs are able to detect all penicillins at 5 or 30 ng/ml (LOD;

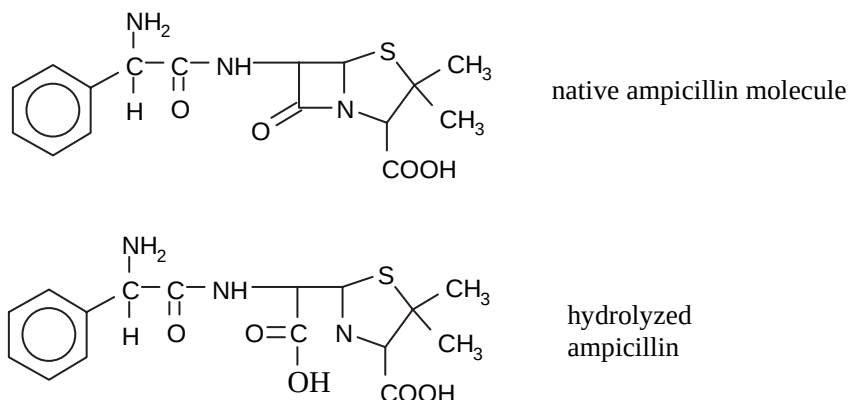


Figure 2. Structure of the hydrolyzed and native ampicillin molecule.

Table IV without hydrolysis). As the antibody cELISA was more sensitive for all three antisera than the antigen cELISA, the first one was chosen for further study.

Physiological penicillin-protein conjugates were used to induce the pAbs. In such conjugates, the β -lactam ring of the penicillins is open (Katsutani & Shionoya 1993). Therefore, it could be expected that the pAb have a higher affinity for the open ring structure of penicillins. Antibodies elicited by a certain conformation of the antigen are extremely sensitive to this form of the antigen (Jemmerson 1995). The β -lactam ring can hydrolyze spontaneously when dissolving penicillins in an aqueous solution or can be hydrolyzed enzymatically with the enzyme Penicillinase I (Boison 1995; Kheirulomoon et al. 1999). Even cloxacillin, which is considered poorly hydrolyzed by penicillinase from *B. cereus* (Bush et al. 1995), can be hydrolyzed by this enzyme (one unit for 1 μ g of cloxacillin) within 30 min (Fink et al. 1987; Grubelnik et al. 2001). The structure of the native and hydrolyzed ampicillin molecule is shown in Figure 2.

To investigate the influence of the hydrolysis of penicillins on the recognition by the antibodies in the antibody cELISA, buffer dilutions of hydrolyzed and non-hydrolyzed penicillin were tested for each penicillin using the three pAbs (Tables II and IV). Two methods for hydrolysis were compared-spontaneous hydrolysis by incubating a penicillin solution overnight at room temperature and enzyme-mediated hydrolysis. Care was taken while preparing the samples containing non-hydrolyzed penicillins to avoid spontaneous hydrolysis; the penicillins were dissolved in buffer just before the samples were added to the ELISA. From the results, it is clear that the hydrolysis of the sample penicillins strongly improved the sensitivity of the assay. The enzyme-mediated hydrolysis is even more efficient than the spontaneous hydrolysis. The LOD of all tested penicillins in buffer solution analyzed after enzyme hydrolysis is lower than 5 ng/ml or 30 ng/ml in each antibody cELISA (Table IV), except for the detection of amoxicillin with pAb K6.

The same approach was followed by Grubelnik et al. (2001). They developed ELISAs for the detection of benzylpenicillin, resp. cloxacillin using enzymatic hydrolysis (with Penicillinase I) and antibodies specific for the hydrolyzed form of the antibiotics. Hydrolysis of the antibiotics induced a shift of the IC₅₀-value from 3.5 to 0.2 ng/ml in the case of benzylpenicillin, and from 2 to 0.65 ng/ml in the case of cloxacillin. In our ELISAs, the impact of hydrolysis was more radical; using the pAb K2 for example, a shift of the IC₅₀-value from 450 to 7 ng/ml in the case of

Table V. Comparison of the IC₅₀-values for six penicillins obtained for antisera pAb K2 and pAb K8 (hydrolyzed, antibody cELISA), and rabbit antisera described in literature.

Antiserum	pAb K2	pAb K8	pAb Grubelnik ^a	pAb Grubelnik ^a
Immunogen ^b	amp-bsa, bpg-bsaIC ₅₀ - values (ng/ml)	amp-bsa, bpg-bsa, ox-bsa, diclox-bsa	bpg-bsa	clox-bsa
amp ^c	0.08	2	4.6	>10 ⁵
amox	2	3.5	2,500	>10 ⁵
bpg	7	0.8	0.2	>10 ⁵
ox	6	10	3,000	29
clox	50	10	140	0.65
diclox	200	6	3,000	105

^aGrubelnik et al. (2001); ^bpen-bsa, physiologic penicillin-bovine serum albumin conjugate; amp-glut-bsa, ampicillin coupled to bovine serum albumin using glutaraldehyde as linker molecule; ^camp, ampicillin; amox, amoxicillin; bpg, benzylpenicillin; ox, oxacillin; clox, cloxacillin; diclox, dicloxacillin.

benzylpenicillin, and from 800 to 50 ng/ml in the case of cloxacillin. Grubelnik et al. (2001) used 20 to 100 times more Penicillinase I for hydrolysis compared to our procedure. The difference in the effect of hydrolysis was probably due to the different concentration range at which the antibiotics were hydrolyzed.

Thus, for the three antisera pAb K2, pAb K6 and pAb K8, the detection of all penicillins in buffer solutions below the MRL in the antibody cELISA was achieved when the penicillins were hydrolyzed with Penicillinase I. In Table V, a comparison is made between the antisera pAb K2 and pAb K8 and the antisera obtained by Grubelnik et al. (2001). With the antisera pAb K2 or pAb K8, lower IC₅₀-value and thus higher sensitivities were obtained for all tested penicillins compared to the properties of the antisera described by Grubelnik et al. (2001), except for cloxacillin. This can be explained by the fact that in this study the rabbits were not immunized with a cloxacillin conjugate. The antisera of Grubelnik et al. (2001) are clearly more specific for the penicillin used as hapten. Thus, the broad specificity and sensitivity of the antisera pAb K2 and pAb K8 can be ascribed to the alternate administration of different physiological penicillin-protein conjugates for immunization.

Strasser et al. (2003) with their antisera developed a sensitive test system for the detection of the intact penicillin structure in milk. The MRL is set up for this structure. With the antisera described in the present study, a sensitive method is developed that mainly detects the degradation products, which are not taken into account in the MRL. However, the detection of the hydrolyzed penicillin can be important. Although no scientific evidence that people can have adverse reactions after intake of penicillin through food products is provided, the risk in the case of previously sensitized individuals may not be underestimated (Ormerod et al. 1987; Dewdney et al. 1991). Ormerod et al. (1987) reported the induction of adverse reactions after the intake of milk containing benzylpenicillin (6 ppb) by individuals who were already hypersensitive for penicillins. De Baere et al. (2002) detected high amounts of metabolites after administration of amoxicillin to pigs, in contrast to what is usually assumed. Furthermore, spontaneous hydrolysis of penicillins will occur during sample handling and will always give an underestimation of the penicillin concentration in the original sample. The MRL for penicillins was one of the first

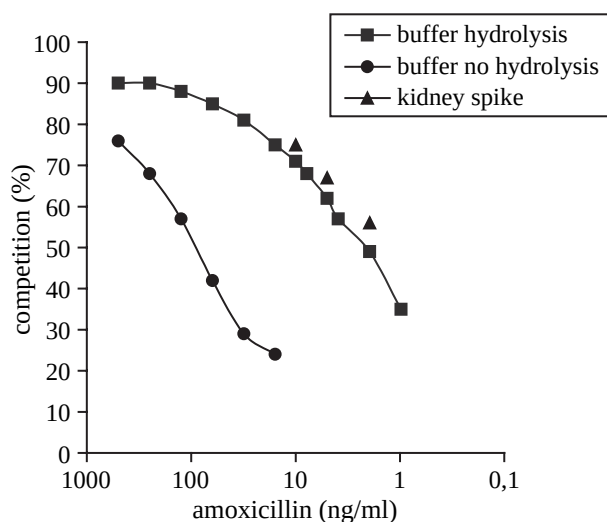


Figure 3. Calibration curve of amoxicillin in buffer solution (hydrolyzed or not) and spiked porcine kidney samples (hydrolyzed), analyzed in the antibody ciELISA with pAb K2.

established levels (Anonymous 1990). At that time, no attention was paid to the metabolites of the molecule. Phillips et al. (2004) remarked that penicillin would nowadays probably not be approved for use in human medicine given its neurotoxicity, the high incidence of allergy, and the common occurrence of resistance.

Sample preparation

Penicillins are present in the intercellular fluid of tissues (Divers 1996). Therefore, tissue fluids were used for analyzing samples in the antibody cELISA with pAb K2. The use of tissue fluid simplifies the sample preparation and analysis. Before analysis in ELISA, the fluids were treated with kaolin. Kaolin treatment reduces the background signals in ELISA by removing disturbing fat or other matrix components (Van den Broeck et al. 1999). A calibration curve prepared using spiked kidney tissues is identical to a calibration curve prepared using buffer solutions (Figure 3). Therefore, when incurred samples are analyzed in the antibody cELISA, buffer solutions of amoxicillin are used as reference. The LOD for amoxicillin in buffer solutions or kidney tissues is lower than 1 ppb (Table VII).

During the extraction procedures, samples are diluted ten times. As a result, the ELISA must be at least ten times more sensitive than the MRL. This requirement was thus fulfilled.

Table VI. Repeatability (Coefficient of variation%) of the antibody cELISA with pAb K2.

	Coefficient of variation (%) for amoxicillin (ppb)					
	40	20	10	5	2.5	1.25
Intra-assay	5–11	5–12	1–13	5–15	6–12	6–14
Inter-assay	0	1	1	2	4	7
Inter day	1	1	2	4	3	4

Table VII. Decision limit ($CC\alpha$), detection capability ($CC\beta$) and limit of detection (LOD) for amoxicillin in kidney samples analyzed in the antibody cELISA.

X_{MRL}	No. of samples B/B_o^a (average $\pm$ SD)	14 0.3300 ± 0.0380
	Competition (average $\pm$ SD)	$67 \pm 4\%$
	Concentration ^d	5 ppb
$CC\alpha$	B/B_o^b	0.2676
	Competition	73%
	Concentration ^d	9 ppb
$CC\beta$	B/B_o^c	0.2052
	Competition	79%
	Concentration ^d	19 ppb
LOD	Concentration	<1 ppb (<56%)

^a B/B_o (X_{MRL}), average absorbance of the sample/average absorbance of the control tissue samples; ^b B/B_o ($CC\alpha$), $B/B_o(X_{MRL}) - 1.64 \cdot SD$ (14); ^c B/B_o ($CC\beta$), $B/B_o(CC\alpha) - 1.64 \cdot SD$ (14); ^dconcentration calculated using the corresponding calibration curve (competition vs. concentration).

Decision limit and detection capability

According to the Commission Decision 2002/657/EC (Anonymous 2002), at least 20 samples should be analyzed to determine $CC\alpha$ and $CC\beta$. The more samples analyzed, the more precise the estimation of $CC\alpha$ and $CC\beta$. In this study, $CC\alpha$ and $CC\beta$ were calculated for the antibody cELISA with pAb K2 using the available data (14 kidneys). The resulting values are, therefore, an estimation of $CC\alpha$ and $CC\beta$. Moreover, the ELISA is a multi-analyte assay for penicillins. The sensitivity is not the same for all analytes. Amoxicillin was chosen as standard analyte. The detection capability and decision limit were established for that standard penicillin. However, the identity of penicillin in a sample is not known during screening. Therefore, decision based on $CC\beta$ (non-compliant if the concentration is higher than $CC\beta$) will not necessarily give certainty about the false compliant rate of the ELISA. Rather, the decision limit and detection capability for amoxicillin are giving information about the precision of the measurements of the ELISA. Since a sample is considered non-compliant when the concentration is higher than the $CC\beta$, compliant when lower than $CC\alpha$ and suspect when between $CC\alpha$ and $CC\beta$, the range between $CC\alpha$ and $CC\beta$ should be as narrow as possible. This is obtained when the repeatability of the ELISA is high (small variations between repeated analyses). For immunoassays, the coefficient of variation should not exceed 15% (Crabbe 2002). This requirement was fulfilled for the ELISA: the highest within-laboratory variation observed was seen in the same assay and was 15% for the antibody cELISA (Table VI).

The ideal group-specific assay would detect all members of the group with the same sensitivity (cross-reactivities 100%). At that moment, a decision based on $CC\beta$ can give a correct idea about the compliance of a sample.

Analysis of incurred samples in the antibody cELISA with pAb K2

To determine the detection capability of the ELISA in accordance with the Commission Decision 2002/657/EC (Anonymous 2002), spiked samples containing penicillin at the MRL should be used. Spiked samples are more appropriate for that purpose than samples obtained from *in vivo* experiments because it is difficult to predict the concentration that will be obtained in the tissues after administration of the

Table VIII. Results of the analysis of incurred kidney samples in the NBKT, the Parallux™ and the antibody cELISA.

Sample No.	NBKT Cortex (mm)	Parallux™ Signal	ELISA Competition (%)
1508	NT ^a	2	74
1770	>2/ >2	2	72
1703	2/2	0	0
1390	>2/ <2	2.03	62
1109	>2/ >2	2.48	76
407	>2/ >2	0	0
1199	4.5/7.6	ceft ^b	12
1388	>2/ >2	2.3	73
1393	<2/ <2	2.07	55
1511	>2/ >2	2.63	66
1408	<2/ <2	0	0
1332	<2/ <2	2.57	66
1679	>2/ >2	2.5	74
1329	<2/ <2	0.77	30
454	>2/ >2	0	0

^aNT, not tested; ^bceft, the suspected analyte was identified as ceftiofur with LC-MS/MS.

drug to animals. On the other hand, care must be taken when validating a method using spiked samples. An extraction protocol optimized using spiked samples will not necessarily be efficient for incurred samples. The efficiency of the extraction procedure to extract analytes from the tissue matrix cannot be determined using spiked samples because the interaction between analyte and the sample matrix is different for incurred samples (McCracken et al. 2000). Therefore, the analysis of incurred samples in the antibody cELISA with pAb K2 was compared to the analysis of the same samples using the microbiological kidney assay (NBKT) and the Parallux™ assay.

Although developed for the detection of antibiotics in milk (Huth et al. 2002), the Parallux™ assay was found suitable for the qualitative screening of different antibiotics (tetracyclins, β -lactams and cephalosporins) in porcine and bovine kidneys (Okerman et al. 2003). The Parallux™ and ELISA detect ampicillin, amoxicillin, benzylpenicillin, cloxacillin and dicloxacillin. The ELISA also recognizes oxacillin.

The results of the analysis of incurred samples in the NBKT, the Parallux™ assay and the ELISA are given in Table VIII. Not only penicillins, but other substances with antimicrobial activity are detected in the NBKT. As the incurred samples came from animals with unknown treatment, a positive result in this test indicates the presence of one or more antimicrobial substances, thus not only penicillins. This is the reason why some samples (sample Nos. 407, 454) are tested positive in the NBKT, but negative in the Parallux™ and the ELISA. High correlation ($r^2 = 0.94$) was found between the Parallux™ and the ELISA. Only sample No. 1199 was tested as negative for the presence of penicillins in the Parallux™, while it was positive in the NBKT and slightly positive in the ELISA (although far below CC α , 73%). However, it was found positive for ceftiofur in the Parallux™. It is possible that the ELISA also detected ceftiofur. The ELISA shows some cross-reactivity toward the structurally related cefaclor. However, cross-reactivity for ceftiofur was not tested.

Acknowledgements

This study was supported by the Ministry of Agriculture, Brussels, Belgium and by the Institute for Veterinary Inspection (Now integrated in the Federal Agency for the Safety of the Food Chain). The technical assistance of Els De Vogelaere was greatly appreciated.

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