Detection of antimicrobial residues in camel milk – suitability of various commercial microbial inhibitor tests as screening tests

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The applicability of six commercially available inhibitor tests for camel milk was determined in a comparative study. 259 milk samples collected from untreated camels were tested using the Delvotest SP and various versions of the brilliant black reduction test (BR-Test "AS Special", BR-Test "AS Brilliant", BRT-Inhibitor Test, BRT-Inhibitor Test with prediffusion and BRT MRL-Screening Test). The occurrence of unspecific reactions (specificity) and the sensitivity against common antimicrobial compounds were assessed. According to German Official Methods of Analysis L 01.00-11 and L 01.00-51-EG the BRT-Inhibitor Test with prediffusion indicated negative results for all samples when the control milk turned yellow, the BR-Test "AS Special" resulted in an earlier colour change. Four test systems (BR-Test "AS Brilliant", BRT-Inhibitor Test, BRT MRL-Screening Test and Delvotest SP) indicated positive – unspecific – results when the tests were evaluated, at the same time the negative control sample showed a complete colour change. However, extension of the incubation period led to negative results for 100 % (BRT-Inhibitor Test and Delvotest SP), 95 % (BR-Test "AS Brilliant") and 89 % (BRT MRL-Screening Test) of the samples, respectively. The sensitivities against most of the antimicrobials tested in camel milk did not significantly differ from those determined in cow milk.

Nachweis von antimikrobiellen Rückständen in Kamelmilch – Anwendbarkeit verschiedener kommerzieller mikrobiologischer Hemmstofftests als Suchtests

In einer vergleichenden Studie wurde die Anwendbarkeit von sechs kommerziellen Hemmstofftests zur Untersuchung von Kamelmilch überprüft. In verschiedenen Versionen des Brillantschwarz-Reduktionstests (BR-Test „AS Special", BR-Test „AS Brilliant", BRT-Hemmstofftest, BRT-Hemmstofftest mit Vordiffusion und BRT MRL Suchtest) sowie im Delvotest SP wurden 259 Milchproben unbehandelter Kamele untersucht. Bei den überprüften Parametern handelte es sich um das Auftreten unspezifischer Reaktionen (Spezifität) sowie die Sensitivität gegenüber verschiedenen, häufig verwendeten Antiinfektiva. Gemäß Methoden L 01.00-11 und L 01.00-51-EG der Amtlichen Sammlung von Untersuchungsverfahren zeigte ein Testsystem (BRT-Hemmstofftest mit Vordiffusion) negative Ergebnisse zum dem Zeitpunkt, an dem die negative Kontrollmilch einen vollständigen Farbumschlag aufwies. Für ein System wurde ein kompletter Farbumschlag sogar früher festgestellt (BR-Test „AS Special"). Vier Testsysteme (BR-Test „AS Brilliant", BRT-Hemmstofftest, BRT MRL-Suchtest und Delvotest SP) zeigten positive – unspezifische – Reaktionen bei Auswertung zur gleichen Zeit wie die Negativkontrolle. Allerdings konnten durch Verlängerung der Inkubationszeiten eindeutig negative Ergebnisse für 100 % (BRT-Hemmstofftest und Delvotest SP), 95 % (BR-Test „AS Brilliant") bzw. 89 % (BRT MRL-Suchtest) der vormals positiven Proben herbeigeführt werden. Zwischen den Sensitivitäten in Kamel- und Kuhmilch gegenüber üblichen Antiinfektiva wurden keine signifikanten Unterschiede für die jeweiligen Testsysteme ermittelt.

38 Camel milk (detection of antimicrobial residues) **38 Kamelmilch** (Hemmstoffnachweis)

1. Introduction

The old world camel population consists of approximately 20 million animals, comprising the one-humped *Camelus dromedarius* and the two-humped *Camelus bactrianus*. With the exception of work or racing use, dromedaries are predominantly kept for their meat and milk in (semi-) arid zones of Africa and Asia (1; 2). In particular, camel milk is gaining increasing importance, as is shown by the establishment of a number of camel farms also in Europe. This can be partly attributed to claims that camel milk components possess medicinal properties suitable for the treatment of several ailments, *e.g.* autoimmune diseases, allergies, juvenile diabetes or skin cancer (3). Additionally, due to its analogy with human milk and the lack of typical cow milk allergens it has been advocated as an appropriate substitute to breast milk (2, 4). Fermented camel milk pro-ducts like (soft) cheese and yoghurt are also quite popular (5; 6; 7). With the intensification of dairy camel husbandry, the prophylactic and therapeutic use of antibiotics is inevitable, thus enhancing the risk of antimicrobial residues in camel milk. In order to ensure the safety of milk for the consumer and the technological quality for the processor, microbial inhibitor tests, based on the growth inhibition of *Geobacillus stearothermophilus* var. *calidolactis*, are commonly applied to detect antimicrobial substances in (raw) cow milk. Whereas some studies have been conducted to investigate the applicability of these test systems for sheep, goat, mare and buffalo milk (8, 9, 10, 11), to our knowledge data concerning camel milk has not yet been reported.

2. Materials and methods

2.1 Camel milk samples

Camel milk samples (n=259) were collected from untreated dromedaries from the camel herd of the Central Veterinary Research Laboratory, CVRL, Dubai. The samples were deep frozen immediately and shipped under cooling conditions to Germany within 1 day, where they were stored frozen until analysis. Prior to testing samples were thawed at 45°C according to (12) and mixed thoroughly. The pH values of the thawed samples were determined by random sampling.

2.2 Microbial inhibitor tests

The microbial inhibitor test systems evaluated comprised of various commercial versions of the brilliant black reduction test – BR-Test "AS Special", BR-Test "AS Brilliant" (both DSM Food Specialties, Delft), BRT-Inhibitor Test, BRT-Inhibitor Test with prediffusion and BRT MRL-Screening Test (all AiM Analytik in Milch GmbH, München) – as well as Delvotest SP (DSM Food Specialties, Delft). The tests were performed according to the manufacturers´ instructions and the German Official Methods of Analysis L 01.00-11 and L 01.00-51-EG for cow milk (13; 14). Inhibitor-free cow milk was used as a negative control and cow milk fortified with penicillin G (4 µg/kg) served as a positive control. All commercial test systems were incubated in a water bath at 64°C ± 1°C. The BRT-Inhibitor Test with prediffusion was incubated at 4°C for 1 h, and milk was discarded prior to incubation in the water bath. The reading of the tests was carried out visually when the negative control sample had developed a yellow colour (2 h - 2 h 45 min or before, if the colour of the camel milk samples changed earlier). Camel milk samples developing the same colour change as the negative control at that time were considered „negative", those showing no or just a partial colour change „positive". For these latter samples the incubation time was prolonged until the test indicator turned yellow or the tests were cancelled after a maximum time extension of 90 min.

2.3 Inactivation of heat-labile inhibitors

In order to differentiate heat-labile from heat-stabile natural inhibitors approximately 10 % of the samples, which were termed "positive" using the majority of tests, were retested. The samples were heated at 80°C for 10 minutes according to IDF [15] in order to inactivate heat-labile inhibitory milk compounds and applied to the six commercial inhibitor tests under study.

2.4 Determination of sensitivity

In orientating experiments the sensitivity of the six commercial inhibitor tests against 7 commonly used antiinfectiva has been determined. Eight camel milk samples were pooled and – as well as cow milk – spiked with different concentrations of penicillin G, cloxacillin, ceftiofur, oxytetracycline, sulfamethazine and gentamicin. Inhibitor-free cow and camel milk, respectively, served as negative controls. The samples were evaluated according to methods L 01.00-11 and L 01.00-51-EG (13, 14) of the German Official Methods of Analysis.

3. Results

3.1 Sample analysis

The BRT-Inhibitor Test with prediffusion at 4°C gave negative results for all camel milk samples tested (n=190) when evaluated at the time at which the cow milk sample was clearly negative. Using the BR-Test "AS Special" camel milk resulted in quicker reactions compared with cow milk: 99% of the samples developed a yellow colour prior to the negative control (most of them 25 min, some up to 40 min). A different pattern was observed with the BRT-Inhibitor Test, BRT MRL-Screening Test, BR-Test "AS Brilliant" and Delvotest SP. When the reactions were read at the moment of the complete colour change of the negative cow milk control, the colour of several camel milk samples showed intermediate colour tones (neither blue/dark purple nor yellow). According to methods L 01.00-11 and L 01.00-51-EG (13, 14) of the German Official Methods of Analysis these samples had to be considered "positive". The frequency of these positive samples ranged from 8.1% using the Delvotest SP to 75.3% using the BR-Test "AS Brilliant" (Table 1). A prolongation of the incubation time (up to 45 min), however, resulted in a complete colour change of all camel milk samples analysed with the Delvotest SP and the BRT-Inhibitor Test. Using the BR-Test "AS Brilliant" and in the BRT MRL-Screening Test 95 and 89%, respectively, of the camel milk samples developed a yellow colour after an additional incubation time of up to 90 min. The pH values for all samples tested were in the range of 6.27 to 6.63.

Tab. 1: Results for camel milk samples (n=259; BRT-Inhibitor Test with prediffusion and BR-Test "AS Special": n=190) using 6 commercial inhibitor tests evaluated at the moment of complete colour change of the negative cow milk control

Test system	Samples (%) with colour tone		
	yellow	inter-mediate	blue/dark purple
BR-Test "AS Special"	100	0	0
BR-Test "AS Brilliant"	24.7	75.3	0
BRT-Inhibitor Test	70.7	29.3	0
BRT-Inhibitor Test with prediffusion	100	0	0
BRT MRL-Screening Test	53.7	46.3	0
Delvotest SP	91.9	8.10	0

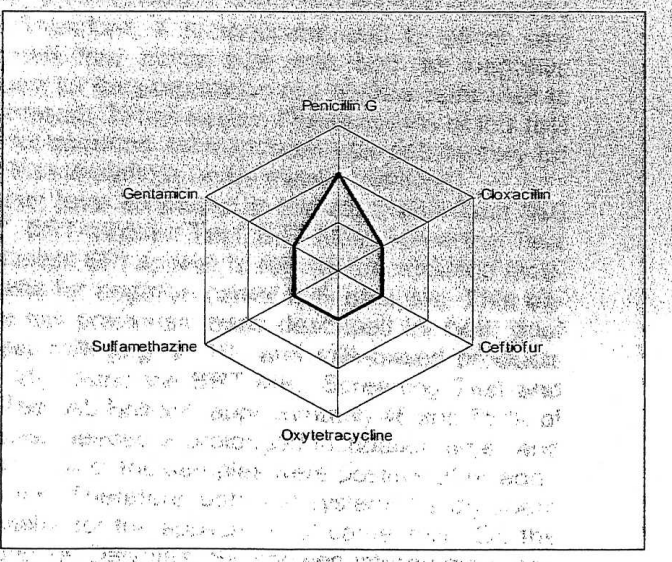

Fig. 1: Sensitivity pattern of the BRT-Inhibitor Test with prediffusion for antimicrobials in camel milk (outer circle 0.5 x MRL, middle circle MRL, inner circle 2 x MRL or higher)

3.2 Inactivation of heat-labile inhibitors

Heat treatment (80°C, 10 min) of samples termed "positive", did not result in any change in test outcomes as the analyses resulted in the same colour tone of the test media as those with untreated samples.

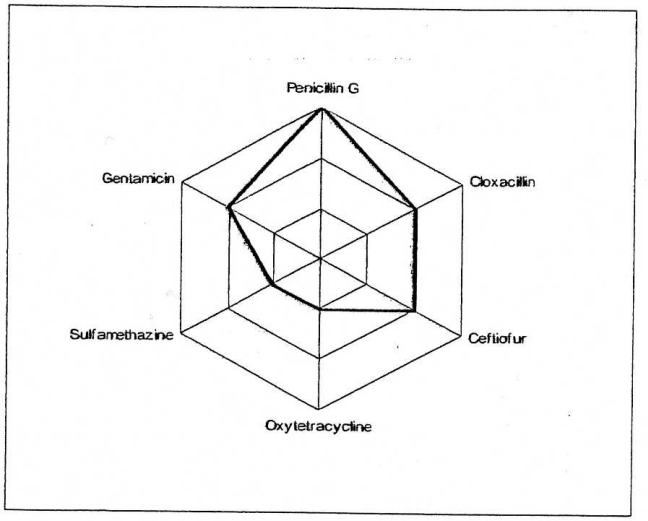

Fig. 2: Sensitivity pattern of the BR-Test "AS Special" for antimicrobials in camel milk (outer circle 0.5 x MRL, middle circle MRL, inner circle 2 x MRL or higher)

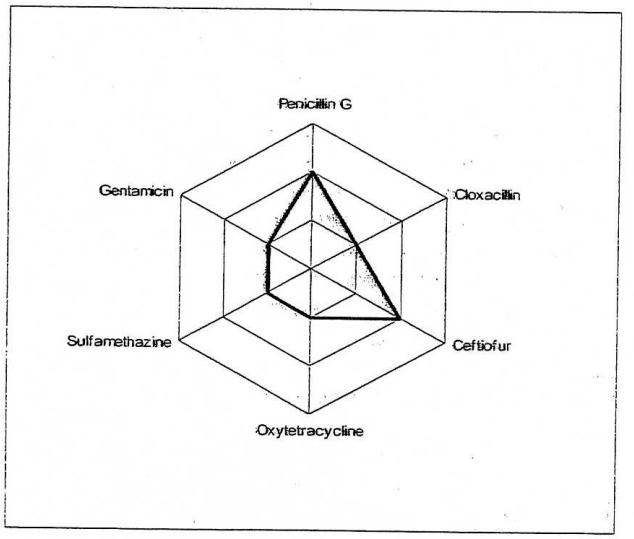

Fig. 3: Sensitivity pattern of the BR-Test "AS Brilliant" for antimicrobials in camel milk (outer circle 0.5 x MRL, middle circle MRL, inner circle 2 x MRL or higher)

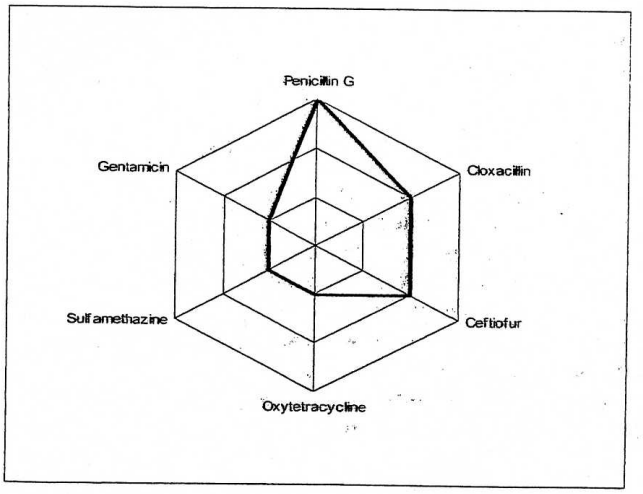

Fig. 4: Sensitivity pattern of the BRT-Inhibitor Test, BRT MRL-Screening Test and Delvotest SP for antimicrobials in camel milk (outer circle 0.5 x MRL, middle circle MRL, inner circle 2 x MRL or higher)

3.3 *Determination of sensitivity*

The determination of sensitivity for seven antimicrobials did not differ significantly from the sensitivities determined for cow milk (data not shown).

In Figs. 1-4 the sensitivity patterns of the six commercial inhibitor test systems under study for camel milk are charted, whereas the results of the Delvotest SP, BRT-Inhibitor Test and the BRT MRL-Screening Test are represented within one graph as these tests showed comparable sensitivities.

4. Discussion

More than 75 % of the 259 milk samples from untreated camels were termed "positive" in at least one test system as they only produced intermediate colour tones – between blue/dark purple and yellow – instead of the true yellow colour exhibited by the negative cow milk control (13; 14).

The BRT-Inhibitor Test with prediffusion, which is also recommended for the analysis of ewe milk [16; 17], was the only test system that showed negative results for camel milk at the same time point as the antimicrobial-free cow milk control developed a clear yellow colour. This might be due to the relatively short contact between the milk sample and the test medium. By discarding the milk after an 1 h prediffusion high molecular substances have not diffused into the medium yet and thus could not interact with the test organism. However, the sensitivity of this system for the tested antimicrobial compounds, with the exception of penicillin G, was not sufficient with respect to MRLs for cow milk.

The BR-Test "AS Special" mostly required significantly reduced test times for camel milk compared to cow milk, a phenomenon, which has not been observed so far. Therefore, it is recommended to either use antimicrobial-free camel bulk milk both as negative control and for the preparation of a positive control or to read the results 30 min earlier. The sensitivity of this test system for common antimicrobials was satisfactory for most of the substances under these conditions.

All other tests employed in the study (BR-Test "AS Brilliant", BRT-Inhibitor Test, BRT MRL-Screening Test and Delvotest SP) appear to require an extended incubation time for negative camel milk samples. This experience has previously been described for both goat and sheep milk [e.g. 9; 10], and milk-based products [18, 19, 20]. Using the BRT MRL-Screening Test and the BR-Test "AS Brilliant" approximately 46 and 75 % of the samples needed a prolonged incubation time, and still up to 11 % of the samples were positive after additional 90 min. Therefore, both test systems hardly seem to be suitable for the application of camel milk. On the other hand the Delvotest SP showed intermediate colour tones for less than 10 % of the samples, thus still implying a quite acceptable reliability towards camel milk. Yet, problems that could arise with differing incubation times for the individual samples may result in over-incubation of the test tablets and lead to false negative results. Instead of applying the German Official Methods L 01.00-11 [13] and L 01.00-51-EG [14], method L 01.01-5 [21] could be applied alternatively for the evaluation. According to this method only those samples are considered "positive", which show the same blue/dark purple colour tone as the positive con-

trol with 4 ppb penicillin G. The application of this scheme to our data would have resulted in no "positives", as none of the samples under study showed these colours. On the other hand, actual antimicrobial residues might remain undetected on account of lower test sensitivities.

The factor(s) partly retarding the growth of the test organism and the complete colour change in the test systems could not be identified. The pH values of 6.27 to 6.63, which were determined for the camel milk samples, fit into the range reported in the literature [2, 22]. However, preliminary experiments showed that these values did not influence the results of the inhibitor tests. The unchanged reactions after heat treatment indicated that heat-labile components, such as lactoperoxidase and immunoglobulins, were also not necessarily responsible for the positive results in the commercial inhibitor tests. Further confirmational tests have not been carried out. A useful tool to withhold inhibiting high molecular compounds from the penetration into the test medium and thus avoiding unspecific reactions would have been the application of a disk assay using a dialysis membrane [23].

According to [24, 25, 26] camel milk possesses a stronger inhibitory system than cow milk, leading to bacteriostatic or bacteriocidal effects against gram-positive and gram-negative strains as well as against starter cultures during fermentation of camel milk [27]. KAPPELER [28] partially attributed these antimicrobial properties to well characterised proteins, such as lactoferrin, lactoperoxidase, lysozyme and immunoglobulin A, which were shown to be present in higher concentrations or possess greater activity in camel milk compared to bovine milk - cow milk contains per ml 0.13-0.7 µg lysozyme and 20-350 µg lactoferrin [19]). Similar findings were made by several authors [24, 29, 30, 31]. In combination, however, lysozyme and lactoferrin inhibited the growth of *Geobacillus stearothermophilus* var. *calidolactis* even at concentrations naturally occurring in cow milk [32], so that the higher concentrations of these substances in camel milk might have been responsible for the reactions in our study. Furthermore, the isolation of another inhibitory protein from camel milk, peptidoglycan recognition protein, has previously been described [28]. It was detected in high concentrations particularly towards the end of lactation, and was capable of inactivating gram-positive bacteria.

5. Outlook

Based on this study, it could be demonstrated that, with certain limitations, commercial inhibitor tests that are predominantly used for screening of (bulk) cow milk, can also be applied to camel milk. Further research is required to find out whether these restrictions also apply to bulk milk samples, where individual variations in milk composition play a minor role, and to determine the mechanisms leading to the unspecific reactions described above (*e.g.* milk composition, stage of lactation, test systems used).

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