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Use of Enzyme Linked ImmunoSorbent Assay (ELISA) for Detection of Antibiotic and Anabolic Residues in Goat and Sheep Meat

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Abstract: Meat samples were collected from Omani and Somali goats and from Australian and Somali sheep at random at Baushar Municipality slaughterhouse, Muscat during April-May 2004. ELISA kits specific for antibiotics and anabolic agents were used to detect residues in the meat. Generally, Meat samples contained various levels of residues of antibiotic and anabolic agents tested with the exception of Sulphamethazene. Tetracycline levels ranged between 44-53 ppb kg^{-1} (mean 49.8); Streptomycin ranged between 0-20 ppb kg^{-1} (mean 11); Chloramphenicol ranged between 0.0-0.08 (mean 0.02). Anabolic agent residues in meat ranged between 0-35, 0-0.05 and 0-016 for Oestradiol 17 β, testosterone and trenbolon respectively. However, levels of these residues were below the international allowable levels set by the German Residue Control Plan (GRCP) and the European Union (EU). Meat from the Omani goat had low levels of antibiotic and anabolic agents compared to other animals, which renders it healthy and safe for consumers. The levels of antibiotic and anabolic residues tended to be lowest in Australian sheep meat and highest in Somali goat meat. This indicates that these animals may have been treated shortly before shipping or after arrival in the country and had been slaughtered before the withdrawal period of the drug expired. Generally, goats had higher levels of antibiotic and anabolic residues than sheep with the difference being significant for tetracycline. This difference was mainly due to the higher levels of these agents in the Somali goat meat. This study indicated that sheep and goat meat sold in Oman generally contains some residues of antibiotic and anabolic agents. Although the levels were within the allowable limits, their presence may still be regarded as a health hazard as they may cause allergic reactions or produce drug-tolerant bacteria in humans. More care is needed to monitor importation regulations of animals intended for human consumption.

Key words: ELISA · meat · residues · hormones · antibiotics · sheep · goat · Oman

INTRODUCTION

Meat is one of the most important constituents of the human diet as it provides protein, energy, vitamins and minerals. However, meat could also become a source of health hazards if it contains excess fat or harmful material such as toxins or residues of chemical agents. Residues in meat may result from many sources such as animal drugs used to prevent or treat diseases or to promote growth; pesticides, feed and agricultural or industrial chemicals [1]. Due to the demand for increasing meat production, several agents have been employed for animal treatment and for growth promotion. These include various types of antibiotics, sulphonamides and synthetic as well as natural anabolic agents. These agents may be potential sources of residues in food, when after administration to animals the withdrawal time, in relation to the maximum residue limit, is not taken into consideration [2, 3]. Antibiotic residues may remain in the animal body for a long time. For instance, 10 weeks after the last injection with penicillin G, residues were present in quantities greater than the allowed maximum residue limits in pigs [3].

Drug tolerance is the maximal level of concentration of residues permitted in animal tissue at the time of slaughter and is intended to ensure that the residue has no harmful effect if ingested [4].

Meat is an important constituent of the Omani people's diet as it is eaten almost every day. Due to short supply of meat from the local market, many live animals

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are imported into the country in addition to large quantities of chilled and frozen meat products. Most of the imported sheep are Australian Merinos and Somali Blackhead. Imported goats are mainly of the Somali goat type.

Recently there has been an increasing international and local awareness of the danger of consuming meat with high levels of drug residues. Many of them are now classified as carcinogenic, toxic or allergenic. Some may also interfere with human and animal natural physiological functions. Therefore, detection of these residues in meat intended for human consumption is very important for the safety of consumers.

This study aimed to detect and quantify antibiotic and anabolic residue levels in meat of local and imported sheep and goats slaughtered at the central slaughterhouse in Oman.

MATERIALS AND METHODS

A total of 40 muscle samples were randomly collected from 10 of each of Omani (Batina) goats, Somali goats, Australian Merino sheep and the Somali blackhead sheep slaughtered at the Central Slaughterhouse, Baushar, Muscat, Oman, during April-May 2004. Both goat breeds and Somali sheep were intact males whereas the Australian Merino sheep were castrated males. The whole right and left *m. psoas major* and *m. psoas minor* were removed from the carcass using a scalpel blade and scissors. They were kept in zipped plastic bags in a chiller (4°C) for 24 h before being stored at -80°C for analyses.

ELISA kits specific for antibiotics and anabolic agents based on an antigen-antibody reaction were obtained from r-Biopharm AG, Germany and stored at 4°C. Enzyme substrate (Urea Peroxide) and Chromogen (tetramethylbenzidine) were added to the wells and incubated.

Frozen samples were thawed at room temperature, fat was separated and meat was ground. Ten to 25 mL of the specific buffer was added to 5-10 g of the ground sample, mixed for 30 min, homogenized and centrifuged at 3000-4000 rpm for 10 min at 15°C. The supernatant layer was decanted and the contents of the flask were dried using a Rotary evaporator and redissolved with the specific buffer. The solution was stored at -20°C in small glass bottles for further analyses. In some analysis, 5 mL of the solution was further purified using a RIDA C18 column. The eluate was then stored at -20°C, for test procedures. Analyses were carried out in duplicates.

Kits were supplied with reagents for the enzyme immunoassay including standards and specific coated micro-titer plates. All reagents in the kit had to be brought to room temperature (23°C) before use. Using a micropipette 50 μL of eluate or sample supernatant (prepared by extraction) was transferred into a glass tube, 450 μL of sample dilution buffer were added and the contents were well mixed on a vortex. Fifty μL of each freshly-prepared standard were added to 450 μL of buffer, supplied with the kit. Fifty μL of each diluted standard solution and each diluted sample of antibody solution was added to each well. The solutions in the micro plate were carefully mixed by rocking the plate manually and the plate was then incubated for 1 h at room temperature. The liquid in the wells was completely removed and wells were washed with distilled water and completely dried. Fifty μL of substrate was added followed by addition of 50 μL of chromogen to each well. The contents were mixed and incubated for 15 min at room temperature in the dark. Finally 100 μL of the stop reagent was added to each well and the plate was cautiously mixed. The absorbance of the color was read within 60 min after addition of stop solution in a Multiskan Spectrophotometer instrument at 405 nm against an air blank.

A Multiskan Ex from Thermo Lab systems was used for reading the micro titer plate. Ascent Software Version 2.6 for Multiskan was used. The measurement mode is continuous and the filter used wavelength was 405 nm. The measurement unit is % absorbance for each concentration of standard and sample. The absorbance value obtained for the standard or sample was divided by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard was thus made to 100% and the absorbance values were expressed as percentage. The % absorbance values, calculated for the standards were plotted in a graph against the concentration of the specific standard of the hormone or antibiotic, which was being estimated. The concentration of the antibiotic or hormone in micrograms/kg (ppb kg^{-1}) corresponding to the absorbance of each sample was read from the calibration curve. To obtain the actual concentration of the antibiotic or hormone in the original sample, the concentration read from the calibration graph was further multiplied by the corresponding dilution factor (this factor is specific for each type of sample) divided by mass of meat. The method described above was the general method of extraction and estimation of antibiotics and hormone residues in meat samples. The antibiotics and the hormones are very unique in their structure, so specific methods and solvents had to be used for each agent. Different buffers were used with various assays as

described in the manufacturer directions manual attached with the kits. These included phosphate-buffered saline (PBS) for streptomycin, testosterone, oestradiol and trenbolon and McIlvain buffer for tetracycline.

The data were subjected to analysis of variance [5] to study the effects of breed, origin and breed/origin of animals using the General Linear Models procedure [6]. Significant differences between treatment means were assessed using the least significant difference procedure at $p<0.05$.

RESULTS AND DISCUSSION

Many types of antibiotics are routinely used for therapeutic and prophylactic purposes in farm animals. Anabolic compounds are used mainly in beef cattle for promoting growth but are not widely used in sheep. Many methods have been used in the past to detect hormonal residues in meat including: uterus weight bioassay, prostate test, thin layer chromatography in urine. However, radioimmunoassay is now considered the most sensitive and accurate and it has been used for this purpose for the past few decades [7]. There are no official detection standards of residues in the Sultanate of Oman. Therefore, maximum levels of acceptable residues used as references in the current study were the GRCP provided in pamphlets with the kits or the EU standards.

Meat samples in the current study contained some levels of residues of all antibiotic and anabolic agents tested (Table 1). The exception was the Sulphamethazene, which was minimal (<1 ppb). In most cases, levels of residues were less than the international levels set by the GRCP and EU.

Tetracycline is an antibiotic widely used in veterinary medicine. In animal species, which are used for food production the residue, its maximum acceptable level was set at 100 ppb by the EU law of drugs. The risks of tetracycline residues in meat include toxic and allergic reactions and disturbance of the consumer's intestinal flora [8]. The range of tetracycline levels detected in the current study (44-53 ppb kg^{-1}) is below the allowable international levels. Limits of quantification for various tetracycline compounds in bovine, swine and poultry muscles were estimated between 1 (chlortetracycline) and 9 ng/g (4-epioxytetracycline) and are well below the tolerance levels set by the European Union [9]. However, the presence of the residues indicates that animals have been treated with the drug and probably not allowed an adequate withdrawal period. Presence of antibiotic residues may be responsible for increasing levels of tolerant bacteria or drug sensitivity in some cases.

Streptomycin is one of the most widely used antibiotics in veterinary medicine. Residues of streptomycin may therefore occur in food of animal origin, if it is not used properly. The Maximum Residue Limit (MRL) for Streptomycin stated by EU regulations is 500 ppb in meat samples. Streptomycin levels in the current study ranged between 0-20 ppb kg^{-1} with a mean of 11 ppb kg^{-1}, which is very low according to EU MRL.

Chloramphenicol is a broad -spectrum antibiotic, which is frequently employed in animal health for its excellent antibacterial and pharmacokinetic properties. However, in humans it may lead to aplastic anemia and a type of blood dyscrasia that is usually fatal at extremely low levels to significant subgroups of the population [10]. Chloramphenicol therefore, has been prohibited for the treatment of animals destined for food production. Chloramphenicol levels in the present study were low compared to the 100 nanograms/kg specified as maximum level.

Sulfonamides are widely used as feed additives for calves, pigs and poultry. They are also used for treating intestinal infections and other systemic diseases. Sulfonamide residues may therefore occur in food of animal origin such as meat and milk. In the US, the tolerance for residues of sulfa in uncooked tissues is 0.1 ppm, and the withdrawal time is 15 days [11]. The EC regulations (No. 675/92) have established a maximum residue limit (MRL) of 100 ppb for sulfonamides in meat. Levels of sulphonamides in meat were negligible as most of the samples contained detection levels of <1 ppb kg^{-1}.

Table 1: Mean, minimum and maximum levels (ppb kg^{-1}) of antibiotic and anabolic residues detected in meat of imported and local sheep and goats in Oman

Agent	Number	Mean	SD	Minimum	Maximum
Tetracycline	40	49.790	2.320	44.000	52.800
Streptomycin	40	10.813	4.980	0	20.000
Chloramphenicol	40	0.022	0.027	0	0.080
Sulphamethazene	40	<1 ppb	NA	0	4.000
Oestradiol 17 β	40	0.080	0.085	0	0.350
Testosterone	40	0.007	0.012	0	0.050
Trenbolon	40	0.017	0.040	0	0.160

SD = standard deviation

Table 2: Mean levels (ppb kg^{-1}) of antibiotic and anabolic residues in meat of imported and local sheep and goats of different species, breeds and country of origin

	Type					
Agent	Australian sheep	Somali sheep	Omani goat	Somali goat	SEM	Effect
Tetracycline	49.600^{a,b}	48.000^{b}	50.560^{a}	51.000^{a}	0.660	*
Streptomycin	6.250^{c}	12.800^{a,b}	10.750^{b}	13.450^{a}	1.344	**
Chloramphenicol	0.026	0.026	0.014	0.022	0.017	NS
Oestradiol 17 β	0.080	0.067	0.039	0.134	0.026	0.08
Testosterone	0.001^{c}	0.007^{b}	0.004^{b}	0.016^{a}	0.004	*
Trenbolon	0.000^{c}	0.002^{b}	0.000^{c}	0.066^{a}	0.009	***

Table 3: Mean levels (ppb kg^{-1}) of anabolic and antibiotic residues in meat from imported and local sheep and goats according to country of origin

	Source				
Agent	Australia	Somalia	Oman	SEM	Effect
Tetracycline	49.600	49.500	50.560	0.522	NS
Streptomycin	6.250^{c}	13.125^{a}	10.750^{b}	0.939	***
Chloramphenicol	0.026	0.024	0.014	0.006	NS
Sulphamethazene					
Oestradiol	0.080	0.101	0.390	0.019	NS
Testosterone	0.001	0.011	0.004	0.003	0.06
Trenbolon	0.000^{b}	0.034^{a}	0.000^{b}	0.011	*

SEM = standard error of means, Means on the same row with no or same superscript do not differ significantly ($p>0.05$)
*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$

Anabolic compounds are frequently used for boosting growth in farm animals especially beef cattle. Oestradiol 17 β is a natural occurring sex hormone but is also available as a commercial growth promotant as Compudose@. The GRCP has specified a legal maximum limit of less than 0.1 ppb (μg kg^{-1}) or 100 ppt (ηg kg^{-1}) in plasma. Although its incidence was below detection limit, oestradiol residues existed in some meat samples with a maximum of 0.350 ppb kg^{-1}.

Testosterone is a natural male sex hormone, which has been used as a growth promotants in its natural or synthetic form (e.g. Ralgro@) mostly in beef cattle. The GRCP has specified a maximum limit of less than 0.1 ppb or 100 ppt in samples. The maximum level detected in the current study was 0.05 ppb kg^{-1}, which is half the allowable detection limit. Trenbolon is a very efficient anabolic steroid with strong androgenic activity. The GRCP has specified a detection limit of less than 10 ppb (10,000 ppt or ng kg^{-1}) for meat samples. Levels of trenbolon in the current study were within the acceptable limits for plasma but there was no available reference for meat.

Growth promotants in the form of anabolic agents as well as others are not widely used in sheep and goats especially those raised on natural grazing pasture such as in Australia and Somalia and obviously they are unheard-of in Oman. This probably explains their low detection levels in this study.

Although the levels of antibiotics and anabolic agents were below standard limits, there were some significant variations between types of animal in the residue content (Tables 2 and 3). Omani goat meat had low levels of antibiotic and anabolic agents compared to other animals, which renders it healthy and safer for consumers. The Australian sheep meat had the lowest tetracycline, streptomycin, testosterone and trenbolon whereas the Somali goat meat tended to have the highest levels of antibiotics and anabolic agents. Somali animals (both sheep and goats) had the highest level of streptomycin compared to Australian and Omani animals (Table 3). This indicates that these animals may have possibly been treated within a short period before shipping or after arrival and being slaughtered without an appropriate withdrawal period from the drug. It is extremely important to monitor imported and local animals intended for slaughter. On the other hand, Australian animals had the lowest antibiotic and anabolic agents, contrary to the public belief that these animals are raised on growth promotants.

Australian sheep were the only castrated males amongst the study groups, which explain the low levels of testosterone in their meat. One finding that was

Table 4: Mean levels (ppb kg^{-1}) of antibiotic and anabolic residues in meat from imported and local sheep and goats of different species

	Species			
Agent	Sheep	Goats	SEM	Effect
Tetracycline	48.800[b]	50.780[b]	0.474	**
Streptomycin	9.525	12.100	1.089	NS
Chloramphenicol	0.026	0.018	0.006	NS
Sulphamethazene				
Oestradiol 17 β	0.074	0.087	0.019	NS
Testosterone	0.004	0.010	0.003	NS
Trenbolon	0.001[b]	0.033[a]	0.008	*

difficult to interpret was that Somali goat meat was the only meat that contained detectable amounts of trenbolon. It is unlikely that this anabolic agent has been used as a growth promotants in Somali goats due to poor management practice and low level of knowledge among traditional livestock owners.

Generally, goats had higher levels of antibiotics and hormones than sheep with the difference being significant for tetracycline (Table 4). This was mainly due to the higher levels of the antibiotics and anabolic agents in the Somali goats. Whether this is a true species difference in the rate of clearing tetracycline and other agents from the body, needs further investigation. The level of clearance is a function of several factors including binding to plasma proteins of tissue structure [12]. Sheep and goats differ in carcass composition with sheep tended to be fatter and goats leaner with higher bone content [13]. Goats also had higher levels of the anabolic agent trenbolon due to the unexplainable higher levels in the Somali goat.

CONCLUSIONS

This study indicated that sheep and goat meat slaughtered and sold in Oman generally contains residues of antibiotic and anabolic agents. Although these levels are within allowable limits, their presence may still be regarded as a health hazard as they may cause allergic reactions or produce drug-tolerant bacteria.

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