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A COMPARISON OF THE SENSITIVITY OF ANTIBIOTIC RESIDUE SCREENING METHODS - THE FOUR PLATE TEST (FPT), THE SCREENING TEST FOR ANTIBIOTIC RESIDUES (STAR), AND THE PREMI®TEST TO SULPHONAMIDE STANDARDS

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ABSTRACT

The sensitivity of three microbiological antibiotic residue screening methods, the FPT, the STAR and the Premi®Test, were compared for the detection of 10 different standards from the sulphonamide (SA) group. Phtalylsulphathiazole (PHT), sulphadimidine (SD), sulphaguanidine (SG), sulphachlorpyridazine (SCHP), sulphamerazine (SRZ), sulphamethoxazole (SMX), sulphanilamid (SAM), sulphanilic acid (SAC), sulphaquinoxaline (SQ) and sulphathiazole (STZ) were tested using the concentrations from 0.05 μg.ml^{-1} to 1 μg.ml^{-1}. The detection sensitivity of the methods represented by minimum inhibiting concentration (MIC) was evaluated. The MIC of SA standards detected by FPT was 0.1 μg.ml^{-1} for SRZ, 0.2 μg.ml^{-1} for SMX, SQ and STZ, 0.3 μg.ml^{-1} for SCHP, and 1 μg.ml^{-1} for SD. No detection sensitivity was observed for PHT, SG, SAC and SAM standards. The MIC of SA standards detected by STAR method was 0.05 μg.ml^{-1} for SCHP, SMX, SQ and STZ, 0.1 μg.ml^{-1} for SRZ, and 0.3 μg.ml^{-1} for SD. No detection sensitivity was observed for PHT, SG, SAC and SAM standards. The MIC of SA standards detected by Premi®Test was 0.05 μg.ml^{-1} for SD, SCHP, SMX, SQ and STZ, 0.1 μg.ml^{-1} for SRZ and PHT, and 0.3 μg.ml^{-1} for SG, SAC and SAM. The MICs represent the detection limit of

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the methods (LOD). The results of examinations showed that Premi®Test is the most sensitive method to sulphonamides followed by STAR method and FPT. Premi®Test detected six SA at the level of the maximum residue limit (MRL) 0.1 µg.ml^{-1} set for SA group, STAR method detected five SA at the level of MRL, and FPT detected only one SA at the level of MRL.

Key words: *detection, FPT, STAR, Premi®Test, sulphonamides*

INTRODUCTION

The occurrence of foreign substances and residues of veterinary drugs in foods of animal origin is systematically monitored from the veterinary and human medicine point of view (Korénková et al., 2006; Marcinčák et al., 2005; Popelka et al., 2001). Residues of veterinary drugs in foods of animal origin represent risks of direct and indirect jeopardy to human health, and they have a negative impact on technological processes in the food industry. From the point of view of the threat to consumer health, antibiotics used in animals are included in the food chain and cause the creation of resistance and allergy (Kožárová et al., 2001; Salem, 1998).

Sulphonamides (SA) are veterinary drugs with wide spectrum of the activity, which inhibit bacteria, chlamydia, toxoplazma and other protozoan agens, especially coccidia in the poultry, rabits and other economic important animal species (Prescott and Baggot, 1993).

Owing to the concern of the residues of SA in food products of animal origin, the current legislation (EEC No. 2377/90; 1990) established the maximum residue limit (MRL) of 0.1 mg.kg^{-1} for SA (all compounds of the SA group) in foods of animal origin.

The MRLs are only of value if backed up with good residue control programmes. The Four Plate Test (FPT) (Bogaerts and Wolf, 1980) as a microbial inhibition test with the test organism *Bacillus subtilis* BGA containing test agar pH 7.2 and trimethoprim (TMP) at a concentration of 0.05 µg.ml^{-1} was widely used for the detection of the presence of SA residues in foods of animal origin in the first stage of residue screening.

The Community Reference Laboratory (CRL) developed a new method, called Screening Test for Antibiotic Residues, for the detection of antibacterial residues in milk and meat. This test is a combination of five plates which are intended to improve the ability of detection of the FPT. A first interlaboratory study was carried out in 1999 with paper discs containing antibiotics and blank samples of meat. A second study was organized with meat samples containing eight different antibiotics and two blank meat samples (Gaudin et al., 2004). For sulphonamides, the test organism *Bacillus stearothermophilus* ATCC 10149 (Test agar DST pH 7.4; TMP at a concentration of 0.005 µg.ml^{-1}) is recommended.

The Premi®Test is based on inhibition of growth of *Bacillus stearothermophilus*, a thermophilic bacterium sensitive to many antibiotics and sulpha compounds. A standardised number of spores are embedded in an agar medium with selected nutrients. On heating to 64 °C, the spores germinate and in the absence of inhibitory substances will multiply, producing an acid. This is visualized as a colour change from purple to yellow using the acid-based indicator Bromocresol purple present in the agar medium. Premi®Test integrated strategy detects antimicrobial compounds at or below the EC MRL in a broad range of food products including meat, eggs, fish and honey (Stead et al., 2004).

However, the post screening verification of the presence of sulphonamide residues in potentially positive samples must be further performed by an integrated test system utilising more specific confirmatory techniques.

The objective of this paper was to determine the most sensitive method from three microbiological antibiotic residue screening tests – the FPT, the STAR method, and the Premi®Test, for the detection of 10 standards from sulphonamide group by the comparison of limit of detection (LOD) of these methods for these drugs. All methods mentioned above are in Slovakia officially approved methods for screening food producing animals and their products for residues of veterinary drugs (Bulletin of the Ministry of Agriculture of the Slovak Republic, part 1, 11, 2004).

MATERIAL AND METHODS

Standard solutions. The stock solution of PHT, SD, SG, SCHP, SRZ, SMX, SAC, SAM, SQ, and

STZ (1000 µg.ml^{-1}) was prepared by dissolving 10 mg of SA standards of SD (Sulfamethazine sodium salt, Sigma S 5637), SG (Sulfaguanidine, Sigma S 8751), SAM (Sulfanilamide, Serva 35670), and SAC (Sulfanilic acid, Serva 35674) in 2.4 ml methanol (Merck, Germany), and SA standards of PHT (Phtalylsulfathiazole, Sigma P 4258), SCHP (Sulfachlopyridazine, Sigma S 9882), SRZ (Sulfamerazine, Serva 35650), SMX (Sulfamethoxazole, Sigma S 7505), SQ (Sulfaquinoxaline sodium salt, Sigma S 7382), and STZ (Sulfathiazole, Serva 35690) in 2.4 ml ammonia (Lachema, Brno), and further diluting to 10 ml with sterile deionised water.

The working solutions of SA were prepared by serial dilutions with sterile deionised water to the concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 µg.ml^{-1}. The working solution with the concentration of 20 µg.ml^{-1} (control sulphadimidine solution) was used to check the quality of prepared agar medium for the FPT, and the working solution of sulphadimidine with the concentration of 1 µg.ml^{-1} (control sulphadimidine solution) was used to check the quality of prepared agar medium for the STAR method. The stock solution of TMP for STAR method was prepared by dissolving 10 mg of trimethoprim standard (Trimethoprim, Fluka 92131) in 1 ml 5 % acetic acid (Merck, Germany) and diluting to 10 ml with sterile distilled water to the concentration of 100 µg.ml^{-1}. The stock solution of TMP was further diluted with sterile distilled water to the concentration of 0.5 µg.ml^{-1}. The stock solution of TMP for FPT was prepared by dissolving 10 mg of TMP standard in 10 ml ethanol (Frukona, Slovakia) and diluting with sterile distilled water to the concentration of 5 µg.ml^{-1}. The stock and working solutions were stored in the refrigerator at +4 °C.

Test organism and test agar. FPT – *Bacillus subtilis* BGA (Merck 10649), Test agar pH 7.2 (Merck 15787), STAR method – *Bacillus stearothermophilus* ATCC 10149 (Merck 1.11499), Test agar DST pH 7.4 (OXOID CM 261).

Preparation of test agar. FPT – test agar was seeded with the test organism *Bacillus subtilis* BGA to obtain the final concentration of 5 x 10^4 spores.ml^{-1} in the agar medium. To obtain the final concentration of TMP in agar medium 0.05 µg.ml^{-1}, 1 ml of TMP solution (5 µg.ml^{-1}) was added to 100 ml of agar medium.

STAR method – test agar was seeded with the test organism *Bacillus stearothermophilus* ATCC 10149 to give a final concentration of 5 x 10^6 spores.ml^{-1} in the agar medium. To obtain a final concentration of TMP in agar medium 0.005 µg.ml^{-1} (1% v/v), 1 ml of TMP solution (0.5 µg.ml^{-1}) was added to 100 ml of agar medium.

Premi®Test kits was obtained from DSM (Delft, The Netherlands).

Testing of sulphonamide standard solutions and reading of results. FPT – filter paper discs (S&S Antibiotic-Assay Discs, Diam ˝ In. Aldrich Z 134104) were moistened with 0.1 ml of SA standard solutions and placed in parallel on the surface of agar medium in the Petri plates. All plates were incubated at 30 °C for 18–24 h. STAR – filter paper discs (Whatman No. 1, diameter 9 mm) were moistened with 0.1 ml of SA standard solutions and placed in parallel on the surface of agar medium in the Petri plates. All plates were incubated at 55 °C for 12–15 h. After incubation, the plates were evaluated and the diameters of clear inhibition zones surrounding the filter paper discs were measured in millimetres. The lowest concentrations of SA standards which inhibited the growth and multiplication of test microorganisms were determined. The lowest concentration of SAs was recorded as the minimum inhibiting concentration (MIC). The minimum acceptable ring zone diameter for the control sulphadimidine solution set by the both reference methods was 5 mm.

Premi®Test – SA standard solutions in a volume of 100 µl were transferred onto the agar in the ampoule of Premi®Test. The ampoules were preincubated for twenty minutes at the room temperature. After preincubation the ampoules were incubated for approximately three hours at 64 °C ± 1 °C in the water bath, and the colour change was evaluated. The results were red after the evaluation of 2/3 of the height of the agar. A yellow colour indicated the absence of SA residues and the purple colour indicated the presence of SA residues at or above the LOD. As a control, 100 µl of distilled water was used.

RESULTS AND DISCUSSION

The mean diameters of the inhibition zones (IZ) produced by residual concentrations of sulphonamide standards detected by FPT and STAR method are presented in Tables 1 and 2. The positive and negative results obtained from the detection of the residual concentrations of SA standard solutions by Premi®Test are summarized in Table 3.

The sensitivity of FPT with the test organism *Bacillus subtilis* BGA to the residual concentrations of the SA standards is presented in Table 1. The MIC of SA standards detected by the FPT was 0.1 μg.ml^{-1} for SRZ, 0.2 μg.ml^{-1} for SMX, SQ and STZ, 0.3 μg.ml^{-1} for SCHP, and 1 μg.ml^{-1} for SD. No detection sensitivity was observed for PHT, SG, SAC and SAM standards.

As Table 2 shows, the sensitivity of STAR method with the test organism *Bacillus stearothermophilus* ATCC 10149 to the residual concentrations of SA standards presented by the production of IZ was different. The MIC of SCHP, SMX, SQ, and STZ was 0.05 μg.ml^{-1}, the MIC of SRZ was 0.1 μg.ml^{-1}, and the MIC of SD was 0.3 μg.ml^{-1}. No sensitivity presented by formation of no inhibition zones was observed for PHT, SG, SAM, and SAC.

The sensitivity of Premi®Test to the residual concentrations of the SA standards is recorded in Table 3. The MIC was 0.05 μg.ml^{-1} for SD, SCHP, SMX, SQ and STZ, 0.1 μg.ml^{-1} for SRZ and PHT, 0.3 μg.ml^{-1} for SG, SAC and SAM. The presented MIC determined the detection limit of the method to sulphonamides mentioned above.

Microbial inhibition tests are highly valuable in the first stage of residue screening owing to their

▼ **Table 1.** FPT (Bacillus subtilis BGA, test agar pH 7.2, TMP 0.05 μg.ml^{-1}) – the mean diameters of the inhibition zones (mm) produced by residual concentrations of sulphonamide standards

▼ **Tablica 1.** FPT (Bacillus subtilis BGA, test agar pH 7.2, TMP 0.05 μg.ml^{-1}) – srednja vrijednost dijametara zone inhibicije (mm) nastale uslijed prisutnosti ostataka koncentracija standarda sulfonamida

Sulphonamides	Concentration of sulphonamides (μg. ml^{-1})						
	0.05	**0.1**	**0.2**	**0.3**	0.4	0.5	**1**
SD	–	–	–	–	–	–	**4**
SRZ	–	**2**	3	4	5	6	7
SCHP	–	–	–	**3**	4	5	6
SQ	–	–	**3**	4	5	6	7
STH	–	–	**4**	5	8	9	10
SMX	–	–	**3**	4	6	8	10
SAC	–	–	–	–	–	–	–
SAM	–	–	–	–	–	–	–
SG	–	–	–	–	–	–	–
PHT	–	–	–	–	–	–	–

Legend: bold numerals present the lowest diameters of the inhibition zones representing the MIC of respective sulphonamide standards/ masno otisnute brojke označuju najmanji promjer zone inhibicije i predstavljaju minimalne inhibicijske zone pojedinih standarda sulfonamida

▼ **Table 2.** STAR (Bacillus stearothermophilus ATCC 10149, test agar DST pH 7.4, TMP 0.005 μg.ml^{-1}) – the mean diameters of the inhibition zones (mm) produced by residual concentrations of sulphonamide standards

▼ **Tablica 2.** STAR (Bacillus stearothermophilus ATCC 10149, test agar DST pH 7.4, TMP 0.005 μg.ml-1) – srednja vrijednost dijametara zone inhibicije (mm) nastale uslijed prisutnosti ostataka koncentracija standarda sulfonamida

Sulphonamides	Concentration of sulphonamides (μg. ml^{-1})						
	0.05	**0.1**	0.2	**0.3**	0.4	0.5	**1**
SD	–	–	–	**2**	3	4	5
SRZ	–	**3**	4	5	6	7	9
SCHP	**1**	4	7	10	10	11	12
SQ	**2**	4	8	9	10	11	13
STH	**4**	6	8	11	12	13	15
SMX	**5**	6	8	9	10	11	13
SAC	–	–	–	–	–	–	–
SAM	–	–	–	–	–	–	–
SG	–	–	–	–	–	–	–
PHT	–	–	–	–	–	–	–

Legend: bold numerals present the lowest diameters of the inhibition zones representing the MIC of respective sulphonamide standards/ masno otisnute brojke označuju najmanji promjer zone inhibicije I predstavljaju minimalne inhibicijske zone pojedinih standarda sulfonamida

▼ **Table 3.** The sensitivity of the Premi®Test to the residual concentrations of sulphonamide standards
▼ **Tablica 3.** Osjetljivost Premi®Testa na koncentracije ostataka standarda sulfonamida

Sulphonamides	Concentration of sulphonamides (μg.ml^{-1})						
	0.05	**0.1**	0.2	**0.3**	0.4	0.5	1
SD	+	+	+	+	+	+	+
SRZ	–	+	+	+	+	+	+
SCHP	+	+	+	+	+	+	+
SQ	+	+	+	+	+	+	+
STH	+	+	+	+	+	+	+
SMX	+	+	+	+	+	+	+
SAC	–	–	–	+	+	+	+
SAM	–	–	–	+	+	+	+
SG	–	–	–	+	+	+	+
PHT	–	+	+	+	+	+	+

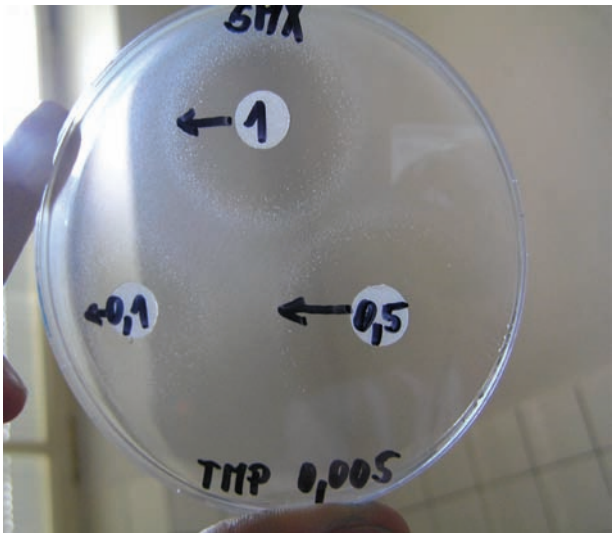
Legend: bold numerals present the lowest diameters of the inhibition zones representing the MIC of respective sulphonamide standards/ masno otisnute brojke označuju najmanji promjer zone inhibicije i predstavljaju minimalne inhibicijske zone pojedinih standarda sulfonamida

excellent practicality and throughput, although they provide only preliminary information about the presence of the residues of certain groups of veterinary drugs in examined samples. The presence of residues presented in the potentially positive samples must be further confirmed by using a more specific physico-chemical method (Ferrini et al., 1997; Kožárová et al., 2001; Kožárová et al., 2002).

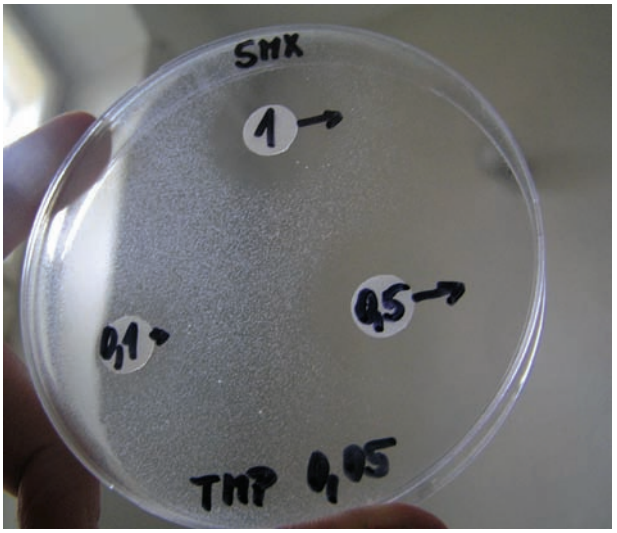
Braham et al. (2001) developed a sulphonamide-sensitive rapid assay using *Bacillus stearothermophilus* inoculated PM indicator agar containing Bromocresol purple and TMP (0.04, 0.05, and 0.12 μg.ml^{-1}), where the end point of the detection was the combination of colour change in the agar medium and zone of microbial growth inhibition around the sample disk. By this method, five sulphonamides were detected at the concentrations near the MRL (from 0.08 to 0.2 μg.ml^{-1}).

Kožárová and Labanská (2005) evaluated the detection sensitivity of Delvotest®SP, Premi®Test, the FPT and the STAR method to six sulphonamides under *in vitro* conditions. They detected that the LOD of Delvotest®SP was for SMX, SQ and SCHP 0.1 μg.ml^{-1}, for SD 0.5 μg.ml^{-1}, and for PHT and SG 1 μg.ml^{-1}, and the LOD of the FPT was for SCHP, SMX and SQ 0.01 μg.ml^{-1}, for SG 0.5 μg.ml^{-1}, for SD 1 μg.ml^{-1}, and for PHT 5 μg.ml^{-1}. The results obtained by the STAR method and the Premi®Test showed the higher sensitivity of these

▼ **Picture 1.** STAR – IZ produced by residual concentrations of SMX standard
▼ **Slika 1.** STAR – zone inhibicije nastale djelovanjem različitih koncentracija ostataka SMX standarda



▼ **Picture 1.** FPT – IZ produced by residual concentrations of SMX standard
▼ **Slika 1.** FPT –zone inhibicije nastale djelovanjem različitih koncentracija ostataka SMX standarda



methods to sulphonamides. Premi®Test detected all sulphonamides tested at the concentrations of MRL 0.1 μg.ml^{-1}, and SCHP even at the concentration of 0.01 μg.ml^{-1}. STAR method detected all sulphonamides tested at the concentration of 0.01 μg.ml^{-1} which is the concentration below the MRL set for sulphonamides. The authors, on the basis of their results, recommended for the primary screening of sulphonamide residues at the levels of the concern only Premi®Test and STAR method.

Bearing in mind these considerations, the aim of our study was to compare the sensitivity of the FPT, the STAR method, and the Premi®Test for the residue screening of 10 different standards from the sulphonamide group. Our results showed that the Premi®Test is the most sensitive method to sulphonamides followed by the STAR method and the FPT. Premi®Test detected six sulphonamides at the level of the maximum residue limit 0.1 μg.ml^{-1} set for sulphonamide group, STAR method detected five sulphonamides at the level of MRL, and the FPT detected only one sulphonamide at the level of MRL.

SAŽETAK

USPOREDBA OSJETLJIVOST SCREENING METODA NA OSTATKE ANTIBIOTIKA - THE FOUR PLATE TEST (FPT), SCREENING TEST NA OSTATKE ANTIBIOTIKA (STAR) I PREMI®TEST NA STANDARDE SULFONAMIDA

U radu je uspoređena osjetljivost mikrobioloških screening metoda u određivanju rezidua 10 različitih standarda sulfonamida. Korišteni su phtalylsulphathiazole (PHT), sulphadimidine (SD), sulphaguanidine (SG), sulphachlorpyridazine (SCHP), sulphamerazine (SRZ), sulphamethoxazole (SMX), sulphanilamid (SAM), sulphanilic acid (SAC), sulphaquinoxaline (SQ) i sulphathiazole (STZ) u koncentracijama od 0.05 μg.ml^{-1} do 1 μg.ml^{-1}.

Minimalna inhibicijska koncentracija standarda utvrđena Four Plate Testom bila je 0.1 μg.ml^{-1} za SRZ, 0.2 μg.ml^{-1} za SMX, SQ i STZ, 0.3 μg.ml^{-1} za SCHP, te 1 μg.ml^{-1} za SD. Minimalna inhibicijska koncentracija standarda utvrđena STAR metodom bila je 0.05 μg.ml^{-1} za SCHP, SMX, SQ i STZ, 0.1 μg.ml^{-1} za SRZ, i 0.3 μg.ml^{-1} za SD. Ni jednom od navedenih metoda nije zabilježena inhibicija za standarde PHT, SG, SAC i SAM.

Upotrebom Premi®Testa minimalna inhibicijska koncentracija bila je 0.05 μg.ml^{-1} za SD, SCHP, SMX, SQ i STZ, 0.1 μg.ml^{-1} za SRZ i PHT, te 0.3 μg.ml^{-1} za SG, SAC i SAM. Minimalne inhibicijske koncentracije predstavljaju prag detekcije metode. Rezultati su pokazali da je najosjetljivija metoda za određivanje ostataka sulfonamida Premi®Test, potom STAR metoda i na kraju Four Plate Test. Premi®Testom utvrđeno je 6 sulfonamida na razini propisane najviše dopuštene količine (NDK) od 0,1 0.1 μg.ml^{-1}, STAR metodom njih 5, a Four Plate Testom samo 1 sulfonamid.

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PROIZVODNJA I KAKVOĆA KOZJEG MESA

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SAŽETAK

Uzgoj koza i konzumacija kozjeg mesa uvjetovani su religijom, tradicijom i običajima, te tržištem i navikama potrošača. Kozje meso je karakterističnog okusa i povoljnog kemijskog sastava, te se preporuča prvenstveno kod zdrave prehrane. U proizvodnji mesa koriste se različite mesnate pasmine poput burske koze te križanci domaće s plemenitim pasminama koza. Pri odgovarajućoj hranidbi postižu dnevni prirast iznad 200 grama, a indeks jarenja je visok i vrlo često po jarenju daju dvoje jaradi. Prema postojećim propisima kozje meso se stavlja u promet kao jaretina i kozetina I. II. i III. kategorije u trupovima, polovicama i četvrtima. Randman se kreće između 35 i 53 posto, a kakvoća mesa određuje na osnovi senzorskih pokazatelja.

Ključne riječi: *kozje meso, kategorizacija mesa, randman, kakvoća*

UVOD

Meso je glavni kozji proizvod prvenstveno u Aziji i Africi, gdje čini više od 90 posto ukupne svjetske proizvodnje. Među vodećim svjetskim proizvođačima kozjeg mesa su Kina, Indija, Pakistan i Nigerija. U Hrvatskoj se za proizvodnju mesa sve više koriste inozemne plemenite pasmine poput burske koze. To je afrička pasmina, a smatra se tipičnom mesnom pasminom radi karakteristične građe, mišićavosti, dobre plodnosti i visokog prirasta. Koze su obično tjelesne mase od 60 do 75 kg, a jarčevi i preko 100 kg. Dnevni prirast u jaradi iznosi 200 – 250 g (Mioč i Pavić, 2002). Osim toga, kod nas se sve više pristupa melioraciji domaće koze s ciljem značajnijeg poboljšanja njezinih proizvodnih osobina. Križanjem domaće s plemenitim pasminama postižu se bolja proizvodna svojstva, tj. brži prirast, bolja konverzija hrane, veća klaonička iskoristivost i kakvoća mesa jaradi, naročito s obzirom na vrlo poželjnu svijetlo ružičastu boju mišićnog i bijelu boju masnog tkiva, poželjnu konformaciju trupa te zadovoljavajuću pokrivenost trupa i bubrega masnim tkivom (Knežević, 1989).

KARAKTERISTIKE MESNIH PASMINA KOZA

Među najvažnije predispozicije koza u proizvodnji mesa ubraja se njihova dobra reproduktivnost

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