Cuboni Reaction: non-invasive late pregnancy test in Martina Franca jennies

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SUMMARY

Martina Franca donkey is an Italian native breed originally from the Puglia region in South Italy. These donkeys are mostly raised in groups that live outdoors throughout the year.

The "Cuboni Reaction" is a test based on a chemical reaction that detects free estrogen in mares' urine using hydrochloric acid, benzene and sulfuric acid; it allows for a late pregnancy diagnosis. To the authors' knowledge, there is only one paper in literature reproducing this technique on asinine species. The purpose of this study is to apply the Cuboni Reaction test to the Martina Franca donkey to confirm the effectiveness of this method in asinine species as well.

18 Martina Franca jennies, whose pregnancy has been confirmed through transrectal palpations and ultrasound examinations, were subjected to urine collection to confirm the pregnancy diagnosis by the Cuboni Reaction.

Data collection were taken at G 120, G 135 and G 150, to identify the moment when the reaction becomes positive. Other samples were taken from G 270, every 15 days until a negative result was obtained. Furthermore, the test was carried out on 2 non-pregnant jennies in order to assess reaction specificity.

Two collection techniques were used: collection from spontaneous urination was performed in jennies not accustomed to the medical procedures; and collection from bladder catheterization in animals accustomed to the medical procedures.

This work shows that Cuboni Reaction is over 50% sensitive from 120th day of pregnancy and 100% sensitive from 150th to 300th day. However, the sensitivity of Cuboni Reaction is less than 40% over 315th day of pregnancy; it also loses specificity detecting false negatives from day 315.

Cuboni Reaction, despite having been overtaken by new technologies for pregnancy diagnosis, still proves useful, particularly in Martina Franca donkey farms, where animals are raised in fields and are not used to handling.

This method can still be used as a late pregnancy test in jennies for which the ovulation day is not known but in which there is a supposed date of mating; it is safe for the involved animals, easy to carry out by the breeder and provides results in a short time.

KEY WORDS

Urine collection; animal welfare; pregnancy diagnosis; jennies; chemical test.

INTRODUCTION

Martina Franca (MF) donkey is an Italian native breed, whose breeding originated in the Itria valley (Puglia, South Italy). Traditionally, these donkeys were used for fieldwork, but new technologies have diminished their usefulness[1]. Nowadays they are considered an endangered species by FAO[2;3].

The current resurgence in interest in donkey breeding originates from their usefulness in pet therapy[4] and milk production[1;3]. In Puglia, for the protection of the native species, MF donkeys are mostly raised in groups, composed of 20-25 jennies and 2 jackasses[1], that live outdoors in fields and wooded areas, separated by drywalls, throughout the year. The recovery center of the Puglia region for the MF donkey (Crispiano, Taranto, 40°37'55.3"N 17°16'31.2"E) corresponds to the characteristics listed.

Pregnancy diagnosis is important to ensure a better management of jennies, predict a precise date of delivery and guarantee animal welfare throughout the gestation period. It is generally known that, unlike mares, pregnancy in jennies lasts on average of 371 days[5].

Many diagnostic techniques have been developed for pregnancy detection, from clinical ones like transrectal exploration[6] and ultrasonography (US)[5], to laboratory tests, less invasive and requiring minimum containment for the animals.

Ultrasonography has been used for over 40 years for pregnancy diagnosis in horses and, in the last 20 years, has been also used in donkeys[7].

This method is elective for pregnancy detection in equids, nevertheless it is not always applicable in field conditions.

Furthermore, animals raised in fields may not be used to handling so, both for the animal's welfare and operator's safety, non-invasive techniques should be preferred.

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In particular, MF jennies are raised in groups in which the male is introduced from April to June and are not used in medical procedures.

Among non-invasive techniques, there is the Cuboni Reaction, a late pregnancy test based on a chemical reaction between free estrogen in pregnant mare's urine and sulfuric acid[8]. The Cuboni Reaction was developed to be performed on the equine species, but in literature there are records of experimental trials also on other species, in particular camelids[9]. Recently, Kubatova *et al.*[10] published the preliminary results of the tests on donkeys. To the authors' knowledge, there are no other publications about application of Cuboni Reaction in donkeys.

Some advantages of the Cuboni test are: the minimal invasiveness of this procedure compared to others, stress reduction for the pregnant animal and consequently lower risk of miscarriages; in fact, urine collection can be performed without capturing the animal[10;11].

The method respects animal welfare, as it reduces stress due to the restraint, thus is easily applicable without any risk for safety of both the breeder and the veterinary technician.

Moreover, breeders can easily perform urine collection and the test is performed quickly[10].

The reliability of a test is determined by *sensitivity* (the likelihood of a positive test results in patients known to have the disease) and by *specificity* (the likelihood of a negative test results in a patient known to be free of diseases)[12].

In jennies, the Cuboni test acquires greater reliability from the second 3rd of gestation (approximately 120th day onwards). Before this date incidence of false negatives is too high and data is therefore not reliable[10]. This is due to low levels of estrogen present in small quantities in urine, making it difficult to detect. It is also known that Cuboni Reaction in mares is not effective over the 300th day of gestation due to the decrease in the concentration of estrogen in blood and urine[6].

The choice of using the Cuboni test for pregnancy diagnosis in MF jennies depends on the sensitivity of the subjects to the handling and the breeding method.

The aim of this project is to assess the reliability of the Cuboni Reaction in jennies in the ranges 120-150 days and 300-345 days of pregnancy. During this time frame, it should be possible to observe a change in the test response in most of the animals in order to determine the range of reliability for the Cuboni Reaction in the asinine species.

MATERIAL AND METHODS

The animals involved in the study were housed at the teaching farm of the University of Teramo, located in Chiareto di Bellante (Te) (42°43'36.3"N, 13°46'23.4"E), during the breeding seasons of 2016-2017. Laboratory tests were carried out at the digestibility laboratory of the zootechnics department of the same University.

18 Martina Franca jennies, in good health and free from reproductive diseases, were subjected to estrous cycle monitoring and to artificial insemination (AI). The time of ovulation was detected by US examination. The first pregnancy diagnosis and subsequent controls were carried out by transrectal palpation and US examination[7].

Urine samples were collected to confirm the pregnancy diagnosis through the Cuboni Reaction.

Ovulation day was regarded as G 0 of pregnancy in order to determine the days for collecting samples. Samples were collected at G 120, G 135 and G 150, to identify the moment when the reaction becomes positive. Other samples were taken from G 270, every 15 days until a negative result was obtained.

Two nonpregnant jennies were subjected to collection to evaluate the specificity of this test.

The techniques used for the collection are the following:

- jennies not accustomed to the medical procedures were moved to a clean box to stimulate urination; a telescopic stick, with a container fixed to the tip, was used for a non-invasive collection (Photo 1A);
- in animals accustomed to medical procedures, urine samples were collected by bladder catheterization (Photo 1B): this method, although minimally invasive, is rapid and involves a minimum stress. A bladder catheter was inserted with the animal adequately contained in a gynecological reinforcement; the anogenital area was previously cleaned with water and betadine and the tail bandaged.

The freshly collected urines were filtered in a graduated cylinder and frozen until the time of analysis.

To check that freezing does not affect the test results, some randomly selected samples were collected in double rate. One of the two samples was frozen and the other one chilled, then subjected to analysis.

The reactions were carried out exclusively under a chemical hood. Operators involved in the procedure were adequately equipped with Personal Protective Equipment (PPE).

Photo 1 - A) non-invasive urine collection; B) kit for bladder catheterization.

Photo 2 Left, positive reaction (green); right, negative reaction (brown).

Distilled water was used as blank control to correct any errors related to the analysis procedure. The blank test was carried out in parallel with each set of analysis.

The protocol used for this analysis has been partially modified compared to the original Cuboni method[8], as follows. Briefly, 3 ml hydrochloric acid (HCl) were slowly added to 15 ml of filtered urine in a test tube. After 10 minutes in boiling water, the tube was cooled to room temperature. The mixture, poured through a separating funnel, was added to 18 ml benzene and shaken carefully for 60 seconds. After separation of the two phases, the lower layer was removed and the top one poured into a second separating funnel, shaken carefully with 10 ml of sulfuric acid (H_2SO_4) for 60 seconds and left to settle. The lower layer was transferred into a test tube and incubated in a water bath for 10 minutes at 90° C. After cooling, the test tube was exposed to sunlight: the sample resulted positive if the liquid assumed a greenish reflection, like lubricating oil (Photo 2, left tube). A homogeneous brownish colour instead indicated a negative reaction (Photo 2, right tube).

The results were defined as uncertain when the reflection of the solution was neither green nor brown.

Data analysis was performed using Manova on the entire dataset and post-hoc one-way ANOVAs were performed on first range and second, a was set as 0.05.

The study was approved by University of Bologna Ethical Committee for Animal Welfare (Prot. N. 62128, 23/04/2018).

RESULTS

In this study, 7 out of 18 pregnant jennies were subjected to urine sampling collection by spontaneous urination, while the remaining 11, by bladder catheterization.

Subjects involved in this study gave birth to alive and viable foals; no negative effects have been reported following the urine collection.

Control tests performed on cooled and frozen samples showed that the freezing process does not affect the results of the Cuboni Reaction, in fact, the results were concordant in 100% of cases.

In non-pregnant jennies, test results were always negative.

Cuboni pregnancy test shows 100% positive results between the G 150 and G 300 of gestation. Before the 150th day, tests showed uncertain results: at G 120; 8 out of 18 samples resulted uncertain (44.33%), and only 4 out of 18 at G 135 (22.21%). However, after 300th day, in addition to uncertain samples, the incidence of false negatives also increases (Table 1).

Table 1 - Results of the performed reactions.

Days of pregnancy	Positive results	Negative results	Uncertain results
G 120	55.56% (10/18)	0% (0/18)	44.43% (8/18)
G 135	77.78% (14/18)	0% (0/18)	22.21% (4/18)
G 150	100% (18/18)	0% (0/18)	0% (0/18)
G 300	100% (18/18)	0% (0/18)	0% (0/18)
G 315	38.89% (7/18)	11.10% (2/18)	50% (9/18)
G 330	0% (0/18)	61.11% (11/18)	38.39% (7/18)
G 345	0% (0/18)	100% (18/18)	0% (0/18)

The data analysis performed on the entire data set using MANOVA show that the test on pregnancy is time locked, $p < .00001$ ($F = 28.67$), (Graph 1A). A series of post-hoc ANOVAs were performed on first and second range, which return in both case statistical significance, $p < .00457$ [$F(2;51) = 6$] and $p < .00001$ [$F(2;119) = 6$] respectively, (Graph 1B, C).

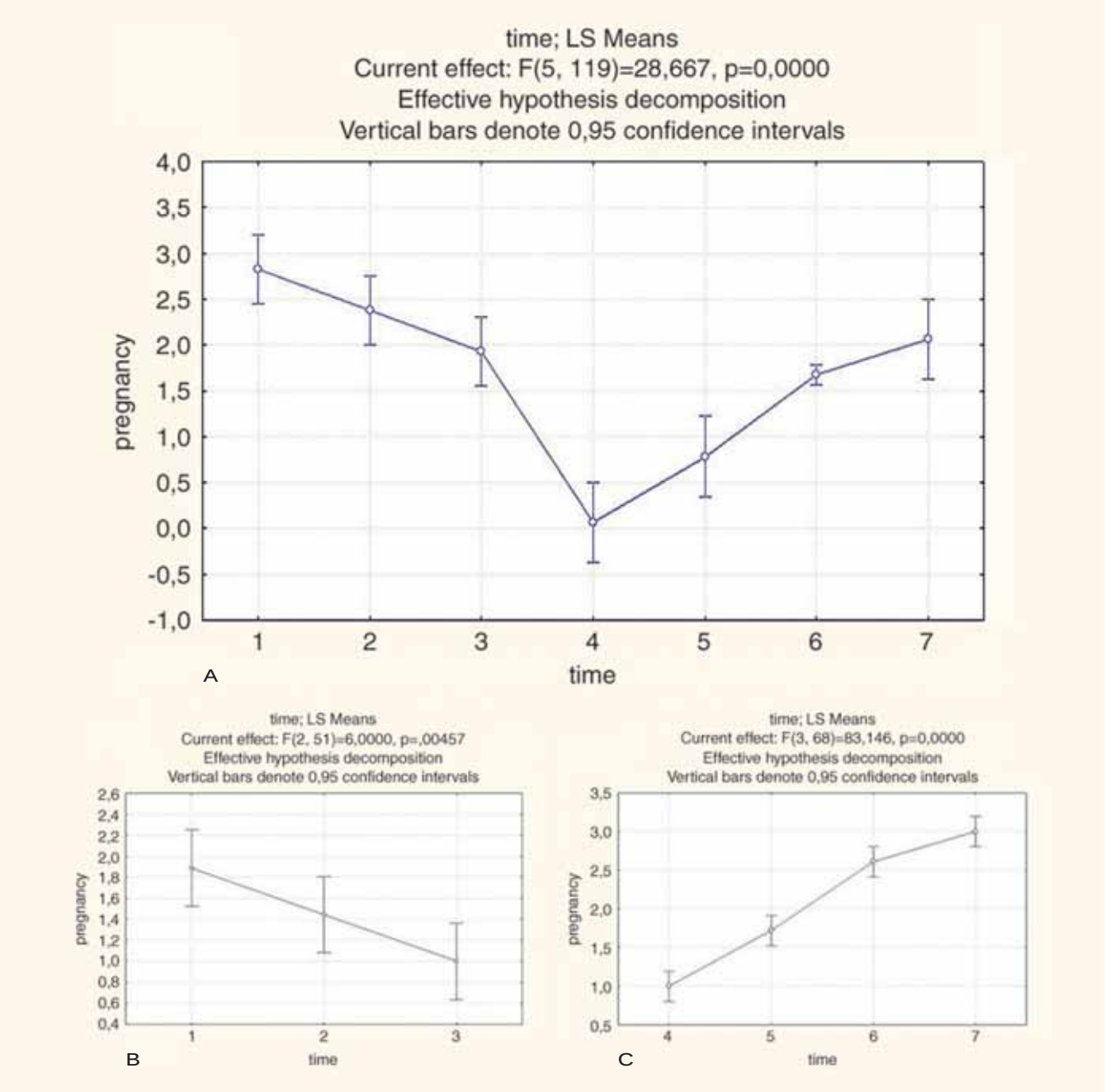
DISCUSSIONS AND CONCLUSIONS

In equine pregnancy, estrogen, whose main source is represented by the foetal gonads, is detectable in the blood from 40th - 60th day of gestation, reaches its maximum values between 150th and 210th days and then decreases rapidly, allowing the preparation for birth[13].

Cuboni Reaction is a chemical test that allows to carry out pregnancy diagnosis highlighting the estrogen in the urine of a mare supposedly pregnant[8].

The bibliography shows that positive results can already be obtained from the 90th - 120th day of gestation[6], but the test becomes reliable from approximately 150th to 300th day of pregnancy[10]. Data presented in this work agrees with other authors, in particular with Kubatova *et al.*[10], who states that the test is 100% reliable in the third period of the gestation.

Furthermore, it is possible to state that the test is not completely reliable before 150th day. In fact, results are uncertain over 40% at G 120 but only over 20% at G 135. Results are clearly positive in 100% of the cases from G 150 to G 300 (Table 1).



Graph 1 - MANOVA analysis was performed on the entire data set (1 = G 120; 2 = G 135; 3 = G 150; 4 = G 300; 5 = G 315; 6 = G 330; 7 = G 345), the test on pregnancy result time locked, $p < .00001$ ($F = 28.67$), (**Graph 1A**). In order, to verify the hypothesis a post-hoc ANOVAs were performed on first and second range (**Graph 1B** and **1C**), both are statistically significant.

The colorimetric reaction is dependent on the concentrations of estrogen and this can cause uncertain results, because, below a given concentration, the colour change to green is not clear enough. Therefore, it is possible to state that Cuboni Reaction is over 50% sensible from 120th day of pregnancy and 100% sensible from 150th to 300th day.

However, over the 300th gestation day, results show that the test loses reliability due to the occurrence of false negatives, never reported before in donkeys. This finding is justified by the decrease in estrogen secretion before birth[14].

In fact, over 10% of false negatives and 50% of uncertain results are detected from G 315 up to 100% of false-negative at G 345 (Table 1).

Hence it can be stated that the sensitivity of the Cuboni Reaction is less than 40% over 315th day of pregnancy; it also loses specificity detecting false negatives after this date.

Test specificity is defined by the negative result in non-pregnant jennies. While Kubatova *et al.*[10] reported over 86% specificity in their work, this work shows 100% specificity. This discrepancy may be related to the smaller control group involved in this work. Moreover, this test proved to be easily applicable both in animals accustomed to human contact and in animals raised in the wild, causing minimal stress in jennies and having no negative effects on pregnancy. In fact, urine collection is less invasive than blood samples or transrectal US.

Another advantage is represented by rapidity in the execution of the test that can also be done in studs provided there is adequate equipment (chemical hood, PPE) for trained operators, such as veterinary technicians.

Furthermore, as revealed by statistical analysis the field test for pregnancy is clearly time locked and return the best score on G 150 and G 300 to identify a pregnant subject, while fail

in G 120, G 330 and G 345, could return unclear results at G 135 and G 315.

In conclusion, Cuboni Reaction, despite being overtaken by new technologies for pregnancy diagnosis, proved to be still useful particularly in Martina Franca donkey farms, where animals are usually raised in fields and are not used to handling. This method can still be used as a late pregnancy test for jennies with an uncertain ovulation day but in which there is a supposed date of mating; it is safe for the animals involved, easily executable by the breeder and provides results in a short time.

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