

Elsevier Editorial System(tm) for Journal of Equine Veterinary Science
Manuscript Draft

Manuscript Number:

Title: The relationship between the positive identification of the embryo proper in equine pregnancies aged 18 to 28 days and its future viability: a field study

Article Type: Original Research

Keywords: mare; embryo proper; early embryonic loss; B-Mode ultrasonography

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Abstract: Early embryonic loss (EEL) negatively affects the reproductive efficiency of equine reproduction. A sign of future EEL is when the embryo proper (EP) fails to develop within the embryonic vesicle after 30 days of gestation. The earlier the identification of impending EEL the earlier the mare can be re-bred to allow a second chance of pregnancy. The objectives of this study were to determine the percentage of embryonic vesicles with a visible EP at 18 to 28 days of gestation and to study the association between the presence/absence of the EP at different days of gestation and the future viability of the pregnancy. A total of 1256 pregnancies were identified and followed by transrectal B-Mode ultrasonography 12 to 45 days post-ovulation in mares of the same Thoroughbred farm. The identification of the EP was attempted once during Days 18 to 28. Pregnancy re-confirmation was performed on Days 35 to 45. The percentage of vesicles with an EP increased gradually from Day 18 (2.8%) to Day 21 (86.9%) $P < .05$. From Day 20 onwards, the EEL rate of mares with vesicles without an EP was significantly higher ($P < .05$) than that of vesicles with a positive identification of an EP. In conclusion, the EP of the equine vesicle can be identified reliably with B-mode ultrasonography in the majority of mares ($> 71\%$) on Day 20 of gestation. The lack of a positive identification of an EP from Day 20 onwards is associated with a higher EEL rate.

Suggested Reviewers:

Opposed Reviewers:

1 The relationship between the positive identification of the
2 embryo proper in equine pregnancies aged 18 to 28 days and its
3 future viability: a field study

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11
12 **ABSTRACT**

13 Early embryonic loss (EEL) negatively affects the reproductive efficiency of equine
14 reproduction. A sign of future EEL is when the embryo proper (EP) fails to develop
15 within the embryonic vesicle after 30 days of gestation. The earlier the identification
16 of impending EEL the earlier the mare can be re-bred to allow a second chance of
17 pregnancy. The objectives of this study were to determine the percentage of
18 embryonic vesicles with a visible EP at 18 to 28 days of gestation and to study the
19 association between the presence/absence of the EP at different days of gestation and
20 the future viability of the pregnancy. A total of 1256 pregnancies were identified and
21 followed by transrectal B-Mode ultrasonography 12 to 45 days post-ovulation in mares
22 of the same Thoroughbred farm. The identification of the EP was attempted once

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23 during Days 18 to 28. Pregnancy re-confirmation was performed on Days 35 to 45.
24 The percentage of vesicles with an EP increased gradually from Day 18 (2.8%) to
25 Day 21 (86.9%) $P < .05$. From Day 20 onwards, the EEL rate of mares with vesicles
26 without an EP was significantly higher ($P < .05$) than that of vesicles with a positive
27 identification of an EP. In conclusion, the EP of the equine vesicle can be identified
28 reliably with B-mode ultrasonography in the majority of mares ($> 71\%$) on Day 20 of
29 gestation. The lack of a positive identification of an EP from Day 20 onwards is
30 associated with a higher EEL rate.

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32 *Keywords:* mare; embryo proper; early embryonic loss; B-Mode ultrasonography

33

34 **1. Introduction**

35 Early embryonic loss (EEL) negatively affects the reproductive efficiency of
36 equine reproduction. It can be defined as pregnancy failure that occurs between
37 fertilization and day 42 of gestation [1]. Although an important number of losses
38 occur between fertilization and day 12 of gestation [1], under field conditions
39 embryonic failure in the mare can only be detected with ultrasound after day 11 to 12
40 of gestation by repeated transrectal examinations. Under these circumstances, the
41 detected incidence of EEL between days 12 and 40 is on the order of 10 to 15% in
42 young mares and 20 to 30% in aged mares [2]. A review on EEL from several
43 controlled and field studies provided a weighted mean of embryo losses between day
44 10 and 60 of gestation of 8.6% (1519 embryo failures of 17581 pregnancies) with a
45 range from 2.6 to 24% [1].

46 Early pregnancy diagnosis is essential for mare management, especially to reduce
47 the need for continued estrus detection. Repeated diagnoses are needed to identify
48 early embryonic failure. The earlier a failure can be discovered, the better the chance

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49 of establishing a new pregnancy, and the least time lost. Prediction that an apparently
50 viable pregnancy could fail would further aid the detection of any failure and reduce
51 the time lost in establishing a new pregnancy.

52 Examination of mares either daily or every two days for ovulation diagnosis allows
53 embryo age to be calculated to ± 12 h or ± 24 h respectively. Examination of the
54 corpus luteum at first detection may further define the time of ovulation and hence
55 embryo age. Embryo development is closely related to embryo age and vesicle
56 diameter at 11 to 15 days post-ovulation, date at which is a good guide to future
57 viability [3]. At this time, the embryonic vesicle grows between 2.9 and 4.5 mm per
58 day [4]. The expected mean diameter of the embryonic vesicle at 13 and 15 days post-
59 ovulation is 12.4 ± 1.8 mm and 20.1 ± 3 mm [4]. At this stage of development, a
60 reduction in diameter equivalent to ≥ 2 days of growth is highly indicative of future
61 embryo failure [3].

62 There is little change in the diameter of the embryonic vesicle from about Day 16
63 to 17, after fixation of the embryonic vesicle at the flexure of the uterine horn [5], and
64 even in viable pregnancies its diameter can vary from under 20 mm to over 30 mm
65 (Newcombe, unpublished data). From about Day 17 onwards therefore, vesicle
66 diameter becomes a less reliable indicator of future viability.

67 Identification of the embryonic disc or 'embryo proper' (EP) with discernible heart
68 beat is often the 'gold standard' for accurate pregnancy diagnosis and embryo
69 viability, especially where endometrial cysts may be confused with an embryonic
70 vesicle or ovulation dates are uncertain. The EP is first visualized with B-Mode (Real-
71 time) ultrasonography on Day 19 to 22 (Day 0 = day of ovulation) [6]. The heart beat
72 can be visualized as soon as the EP is also detected. This can be greatly aided by
73 color-Doppler ultrasonography. In one study, in which color-Doppler was used, the

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74 EP and heart beat were first detected at 19.1 ± 0.1 days of gestation [7]. In the latter
75 study, an early indicator of the future EP development could be identified at the future
76 position of the EP even 3 days earlier (16.4 ± 0.2 days). This was represented by a
77 colored spot, indicating blood flow, in the endometrium at the ventral aspect of the
78 vesicle opposite to the mesometrial attachment and adjacent to the wall of the vesicle
79 [7]. However, under field conditions, the use of simple B-Mode ultrasonography is
80 more common practice.

81 The presence of an embryonic vesicle without the development of an EP
82 (anembryonic vesicle) beyond day 30 of gestation has been considered abnormal and
83 linked to pregnancy failure [8]. The objective of this study was to determine the
84 ability to detect the EP and heart beat under field conditions in a large population of
85 mares between Days 18 and 28 of pregnancy and to study the association between
86 failure to detect the EP at different days of gestation with the future viability of that
87 pregnancy.

88 89 **2. Materials and methods**

90 91 *2.1. Detection of ovulation and post-mating treatment*

92 Over four breeding seasons, 1256 embryonic vesicles from 1109 different mares
93 were detected at routine ultrasonographic pregnancy diagnosis by a single operator at
94 a Thoroughbred (TB) farm in the UK (northern hemisphere). Mares were examined
95 with an ultrasound scanner (DP-6600Vet, Mindray Co., Ltd, USA) equipped with a
96 linear array 5-10 MHz transducer every two days for the detection of ovulation and
97 again if a second ovulation was suspected. The day of ovulation was defined as Day 1
98 and depending on palpation and the appearance of the ovulatory area or early CL on

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99 ultrasound [9], the day of first detection of ovulation was defined as either Day 1
100 (when the ovary with a previously recorded preovulatory follicle presented an ill-
101 defined ovulatory area of low echogenicity with or without a central lacuna containing
102 echoic mobile particles) or Day 2 (when the ovary with the previously preovulatory
103 follicle presented a well defined hyperechoic corpus luteum with or without a central
104 lacuna containing solid strands of fibrin). All mares were mated by natural cover
105 before ovulation by one of five TB stallions of proven fertility ($\geq 65\%$ per cycle
106 pregnancy rate) within the same farm. All mares received the same post-mating
107 treatment consisting of an intrauterine infusion of 12 mL of a mixture of injectable
108 procaine penicillin (1800 mg) suspension (6 mL Depocillin®, Intervet, Cambridge,
109 UK) and 900 mg of framycetin (6 mL of Framomycin® 15% injection, Novartis
110 Animal Health, Camberley, UK) one to two days post-mating followed by a bolus of
111 25 IU of oxytocin (Oxytocin Leo; Leo Pharma Laboratories Ltd, Princes Risborough,
112 UK) administered intravenously one day later.

113 114 *2.2. Pregnancy diagnosis*

115 Initial pregnancy diagnosis was performed on Days 12 to 14. The next examination
116 was performed six to 10 days later (Days 18 to 24) and briefly searched for evidence
117 of a hyperechoic body of 1.5 to 2.5 mm diameter with a discernible heart beat (EP)
118 around the perimeter of the vesicle (**Fig. 1**). Suspect bodies in which the heart beat
119 was not discernible were not included as positive identifications. No more than one
120 minute was spent looking for the EP. All data from pregnancy diagnoses at Days 18 to
121 28 were obtained from single mares, therefore each mare was examined only once
122 between Days 18 and 28 of gestation.

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123 Twin management was performed by manual reduction between Day 13 and Day
124 16. These mares were re-examined either two or four days later. Mares in which on
125 Days 18 or 19, the remaining vesicle appeared normal were not re-examined until 6 to
126 8 days later and were not therefore included in the Day 20 to Day 23 data. Further
127 examinations were made on Days 30 to 32 and 42 to 45 to detect embryo failure.
128 Embryo failure was assumed when the embryonic vesicle was no longer visible
129 ultrasonographically or when the fluid-filled vesicle had failed to developed an EP
130 after Day 30. The fetal stage was defined as a pregnancy of > 42 days that showed a
131 positive heart beat.

132

133 2.3. *Statistical analysis*

134 Binary logistic regression was used to compare the difference in embryo detection
135 rate amongst days of gestation (Days 18 to 28) and to compare the early embryonic
136 loss rates between vesicles with a visible EP and those with no EP at a given day of
137 gestation. The predictive value of detecting an EP within the vesicle at a given day of
138 gestation on the future viability of the pregnancy was performed by estimating the
139 sensitivity and the specificity of the test at each day of pregnancy diagnosis relative to
140 ovulation.

141 The specificity was expresses as the number of vesicles with an EP detected that
142 survived to the fetal stage (true negatives) divided by the number of vesicles with an
143 EP detected that survived to the fetal stage (true negatives) plus the number of
144 vesicles with no EP detected that survived to the fetal stage (false positives).

145 The sensitivity was expressed as the number of vesicles with no EP detected that
146 failed before the fetal stage (true positives) divided by the number of vesicles with no
147 EP detected that failed before the fetal stage (true positives) plus the number of

148 vesicles with an EP detected that failed before the fetal stage (false negatives). The
149 specificity and sensitivity of the identification of an EP diagnostic test at each day of
150 pregnancy (Days 18 to 28) are given in percentages (%).

151

152 **3. Results**

153

154 The overall EEL of the study between Day 18 and 45 was 5.6% (70/1256). The EP
155 detection rate increased gradually ($P < .05$) from Day 18 to Day 21 when it reached
156 the maximum. After that day, the number of vesicles with an EP detected became
157 constant (**Fig. 2**).

158 Embryonic vesicles with an EP detected on Days 20 to 24, 26 and 28 were less
159 likely ($P < .05$) to fail than those with a negative identification of the EP at similar
160 gestational ages (**Fig. 3**). The EEL incidence in vesicles with an EP was not
161 influenced ($P > .05$) by the day of gestation in which the EP was detected (**Fig. 3**). In
162 contrast, the longer in gestation that the vesicle remained without a visible EP, the
163 more likely ($P < .05$) it was to fail subsequently (**Fig. 3**).

164 The sensitivity of the diagnostic test on the future viability of the pregnancy was
165 relatively high (> 92%) throughout the gestation period studied (**Table 1**), in part due
166 to a low number of pregnancy failures resultant from vesicles with a positive
167 identification of an EP at earlier stages of gestation (Days 18 to 28; false negatives).
168 In contrast, the specificity of the test was low on Days 18 to 20 (**Table 1**), because
169 many vesicles with no EP detected on Days 18 to 20 still developed normally to the
170 fetal stage (false positives). The specificity of the test increased substantially from
171 Day 21 onwards.

172

173 4. Discussion

174 Causes of embryo loss are multiple, maternal and embryonic, intrinsic and
175 extrinsic and have been reviewed [10-12]. Previous investigations have shown that
176 early vesicle diameter is well correlated with future pregnancy survival [8,13,14].
177 Small for age (SFA) pregnancies are found in mares of all ages [8,15] and occur more
178 frequently following multiple ovulation [3,16]. Those vesicles most retarded in
179 development may be unable to block luteolysis so that the vesicle is eliminated as the
180 mare returns to estrus [11]. In addition, SFA vesicles are more likely to fail to develop
181 an EP [8].

182 If the time of ovulation is known accurately, then a SFA vesicle can be diagnosed
183 at early stages. Then the failing pregnancy can be terminated to allow return to estrus
184 with the subsequent possibility to establish a new pregnancy as early as possible. A
185 delay in embryonic vesicle diameter equivalent to two or more days' growth has been
186 proposed to be highly indicative of subsequent pregnancy failure [11]. However, most
187 practitioners tend to leave these pregnancies until later stages (25 to 30 days of
188 gestation) to give an "extra chance" of survival. If by then, the vesicle still remains
189 without an EP, the pregnancy can be safely terminated [8]. On some occasions,
190 however, embryo vesicles whose diameters fall within the normal range for their age
191 still fail to develop an EP [8].

192 The results of the current study showed that by Day 20, the majority of embryonic
193 vesicles had an ultrasographically visible EP detected by B-Mode under field
194 conditions. This is in agreement with a previous controlled study in which the EP was
195 first detected on Days 19 to 22 (Day 0 = day of ovulation) [6]. In the current study,
196 after Day 21 of gestation the percentage of new vesicles with an EP did not increase
197 significantly along with gestational age.

198 On Day 20 of gestation there already exists a significant difference between the
199 future EEL rate of mares with vesicles without an EP and those that have. This
200 difference becomes increasingly greater as the age of gestation increases. However, at
201 20 to 24 days of pregnancy, even though there is a positive association between the
202 absence of the EP and future pregnancy loss, it seems not advisable to terminate these
203 pregnancies owing to the low specificity of the diagnostic test. For example, on Day
204 24, the specificity of the test was 50% which means that half of the vesicles without
205 EP detected at that date will still develop one and survive beyond the fetal stage.

206 The low specificity of the test on Days 21 to 24 may be due to two reasons: failure
207 to identify the EP on the ultrasound by the operator; and individual “physiological”
208 variation of mares in the interval between fertilization and the development of the EP
209 to a sufficient size at which it can be reliably detected on ultrasound.

210 From Day 26 onwards, both the specificity and sensitivity are high (> 92%). In
211 fact, all pregnancies on Days 26 and 28 without an EP were lost (specificity of 100%).
212 However these results are based on a relative small number of vesicles (3 pregnancies
213 for each day) and therefore the clinical implications must be interpreted with caution.

214 It seems that the optimum time to perform a second pregnancy diagnosis in mares
215 would be on Day 21 to 22. At that stage of gestation, if an EP is identified within the
216 vesicle, the pregnancy is highly likely to progress to the fetal stage. This is indicated
217 by the high sensitivity of a positive diagnosis of the EP within the vesicle on these
218 days (> 95%). On the other hand, when the EP is not visible, it indicates that the
219 vesicle is more likely to be abnormal and therefore more frequent examinations
220 should be performed to monitor the development of the embryonic vesicle. Probably
221 by around Day 26 to 28, if the vesicle remains without an EP, it can be considered
222 safe to terminate the pregnancy provided that the ovulation date is known accurately.

223 In conclusion, the EP of the equine vesicle can be identified reliably with B-mode
224 ultrasonography in the majority of mares (> 71%) on Day 20 of gestation. The
225 percentage of vesicles with an EP increases significantly from day 20 to 21 and then it
226 reaches a plateau. The lack of a positive identification of an EP from Day 20 onwards
227 is associated with a higher EEL rate.

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Table 1

Embryo proper detection rate and predictive value of viability of pregnancy by detecting the embryo proper at different stages of early gestation.

Day of gestation	18	19	20	21	22	23	24	25	26	27	28
Pregnancies (n)	70	90	389	245	171	105	48	50	21	36	31
Vesicles with EP (%)	2.8	31.1	71.4	86.9	87.1	90.4	91.7	100.0	85.7	100.0	90.3
Pregnancy losses (n)	2	4	18	13	12	6	3	3	4	0	5
Sensitivity (%)	100.0	100.0	98.2	96.7	98.6	97.9	97.7	94.0	94.4	100.0	92.8
Specificity (%)	2.9	6.4	11.7	18.7	45.4	40.0	50.0	-	100.0	-	100.0

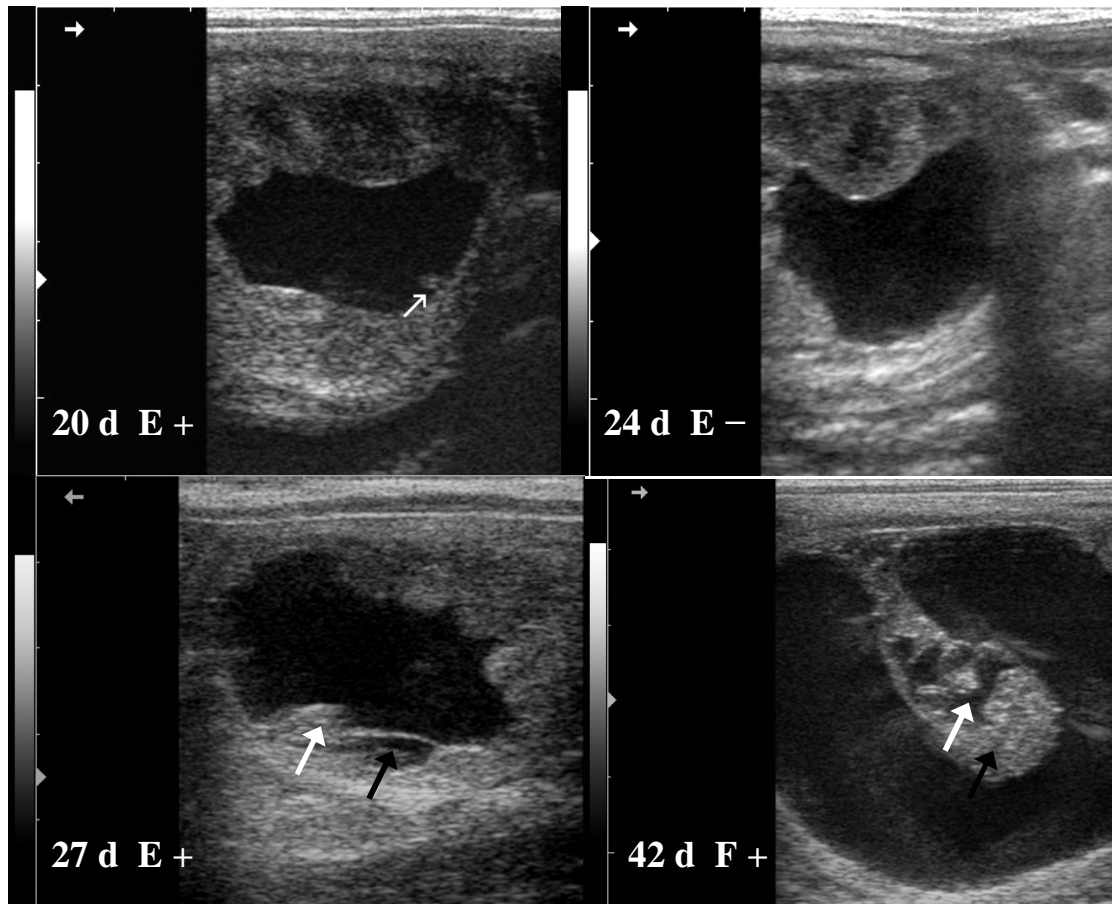


Fig. 1

Figure 2

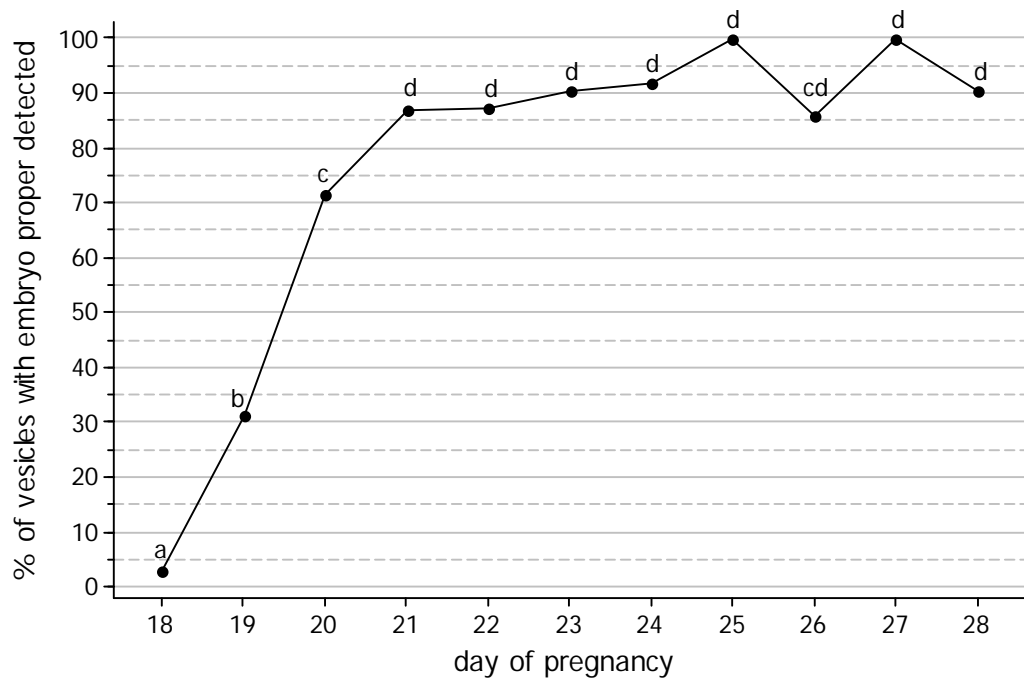


Fig. 2

Figure 3

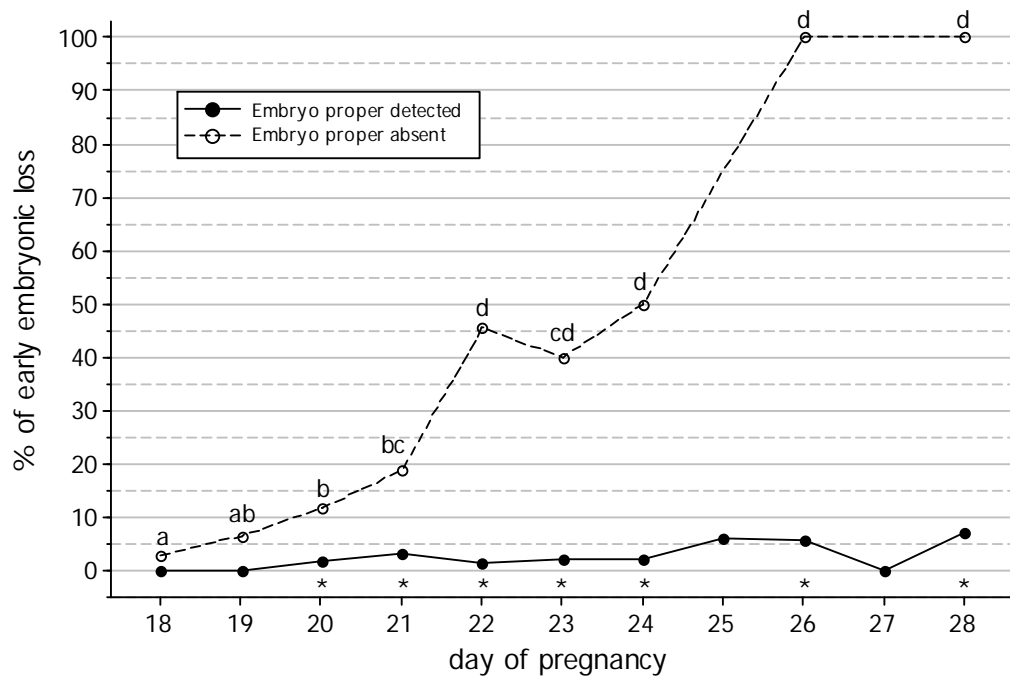


Fig. 3

Fig. 1. B-Mode sonograms of four pregnancies from four mares at different stages of gestation. 20 d E +: pregnancy Day 20 of gestation (Day 1 = day of ovulation); note the embryo proper (EP) at 5 o'clock position (white arrow). 24 d E -: pregnancy Day 24 of gestation; the EP could not be found, this pregnancy failed before day 42. 27 d E +: pregnancy Day 27 of gestation; note the large EP (white arrow) and the developing allantoids (black arrow). 42 d F +: pregnancy Day 42 of gestation; note the developing amnios (white arrow) around the fetus (black arrow).

Fig. 2. Percentage of vesicles with an embryo proper (EP) detected by B-Mode ultrasonography at different stages of early gestation. Significant difference ($P < .05$) in EP detection rate amongst days of gestations is indicated by different letters.

Fig. 3. The association between the ability to detect the embryo proper (EP) within the embryonic vesicle on Days 18 to 28 of gestation (Day 1 = day of ovulation) and the future pregnancy failure before day 42. Within mares with vesicles with no EP, different letters amongst days of pregnancy indicate significant difference ($P < 0.05$) in embryo loss rate. The embryo loss rates in mares with vesicles with an EP were not different ($P > .05$) amongst days of gestation. Within day of gestation, an asterisk (*) indicates a difference ($P < .05$) in the future early embryonic loss rates between mares with vesicles with an EP and those without one.