SOCIETY FOR THERIOGENOLOGY

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SOCIETY FOR THERIOGENOLOGY MANUAL

FOR

CLINICAL FERTILITY EVALUATION OF THE STALLION

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DEDICATION

This manual is dedicated to the memory of Professor W. Bielanski, one of the finest men to have had a major interest in stallions. Professor Bielanski and his proteges have made major contributions to our evolving knowledge of stallion fertility and its evaluation. His dedication to his profession and his fellow workers, often under trying circumstances, is an inspiration to all who knew him.

Professor Bielanski critiqued an early draft of this manual. He was a strong advocate of an "international" manual and would be pleased that the first edition will see the light of day.
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A. CONDUCTING THE EVALUATION

1. Introduction:

The purpose of conducting an examination of a stallion for the evaluation of fertility is to select stallions which can reasonably be expected to be capable of efficiently rendering at least 75% of 40 or more mares pregnant when bred naturally or 120 mares when bred artificially in one breeding season when given reasonably good management and mares of reasonably good fertility; to eliminate those with genetic defects, and to eliminate from consideration for breeding those that can reasonably be expected to be incapable of achieving the above level of fertility, or at least alert owner of potential problem(s). In addition, the examination will assist in identifying the cause(s) of reduced fertility and the findings used to develop guide lines for management of the stallion to enable it to achieve its maximum fertility.

It is realized that this examination may not invariably and reliably predict the level of fertility any particular stallion will achieve under a multitude of management conditions.

Fertility predictions are made on the basis of the findings at the time of examination. We realize intrinsic fertility may vary over a period of time.

The best measure of stallion fertility is the foaling rate achieved with mares of normal fertility under optimal management conditions. The next best estimation is made by comparing the results of thorough physical and semen examinations with the same measures in stallions of known normal fertility.
There is no single physical or seminal parameter which is satisfactorily correlated with fertility of the stallion and the best combination of measures remains to be determined. The procedures and their analyses proposed here represent a comprehensive yet practical approach to achieve the objectives indicated above. Stallions breeding less than 20 mares by natural service or 60 mares by artificial breeding may not need to achieve the levels recommended here. In the following paragraphs are outlined the methods of examination and analysis currently recommended by the Society of Theriogenology. In the discussion which follows some of the weaknesses in current methods are considered.

Appendix 1 provides a sample of the form used for recording the behavioral, physical and semen findings. The listing of the form indicates the array of examinations that are made. Appendix 2 gives the work forms for recording observations on longevity of motility and morphology. The appendix also presents for quick reference the step by step procedures for conducting the various tests as well as a list of materials and their source in the United States.

If there is a need for less stringent criteria, say for a horse that someone wants to breed to 10 mares, or if someone doesn't want or need a thorough exam, then the type of examination and findings should be explicitly stated and the stallion passed as Satisfactory for 10 mares or other stated limitations. It should be reported over the veterinarian's signature.

There follows on the next 2 pages a broad outline of the steps of a fertility evaluation.
2. OUTLINE OF STEPS FOR STALLION FERTILITY EVALUATION

1. Take history, do physical exam and collect blood sample for E.I.A.

2. Prepare A.V. for collection.

3. Bring stallion to vicinity of restrained mare and record time to full erection.

4. Examine, wash and rinse penis including shaft, prepuce, glans, fossa glandis and urethral process. Use warm water and non-residue soap such as Ivory. An example of an undesirable residue is hexachloraphene. Clean smegma off so can examine for skin lesions. Examine glans penis (corona), fossa glandis and urethral process. Record evidence of all abnormalities.

5. Rinse thoroughly with clean warm water from another pail. Sponge dry.

6. Swab urethra in sterile manner (pre-ejaculation swab).

7. Allow horse to approach and mount an estral, restrained mare or phantom

8. Hold penis to side to permit voiding of pre-ejaculatory secretions.

9. Insert penis in A.V. and collect the ejaculate.

10. Keep fingers on ventral shaft of penis to detect urethral pulsations associated with ejaculatory jets.

11. When ejaculation is complete, allow penis to relax, tilt A.V. downward to allow semen to enter collection bag and hand A.V. to assistant.

12. Swab urethra (post ejaculation swab) in sterile manner.

13. Remove collection bag from A.V.

14. Take loop or swab of semen for culture (semen swab).

15. Remove gel (by aspiration).

16. Filter remainder to remove gross debris if necessary, (sterile in-line milk filter; see Appendix 3 for preparation).

17. Promptly estimate initial total and progressive motility (on warm slide under warm coverslip).

18. Promptly measure pH.
19. Take aliquot for determining sperm concentration.

20. a. Make 4 smears for staining sperm and 4 additional smears for detecting presence of WBC and other cells.

   b. If morphological evaluation is to be made under phase microscopy with buffered formal-saline (BFS) preserved sperm, place sperm in BFS at this time. Make smears for cytological evaluation of stained, non-sperm cells.

21. Set up longevity trials with and without extender.

22. Measure sperm concentration.

23. Palpation of internal and external genitalia is best performed between ejaculates. Second best is after the second ejaculate. Record the size and consistency of genital organs.


25. Repeat entire process one hour later starting with teasing of stallion.

26. Interpret.

27. It may be necessary to collect additional ejaculates to evaluate a particular stallion.
3. **Identification and History:**

Fertility evaluation of the stallion is begun by identifying the animal and recording his breed, age and use. Record all identifying marks and tattoos. (Wise for identification purposes to photograph the stallion in 4 views.) It is essential that the examiner be able, at a subsequent date, to make positive identification of the stallion examined.

For problem stallions and for analysis of the problem, the results of the stallion's previous breeding should be obtained. It is often the best "predictor" of fertility. Of particular importance is a measure of his previous fertility in each of 3 groups of mares - the maiden, barren and foaling mares. The ideal measure is the services per foaling. Be aware that this figure can be altered by abortions unrelated to the stallion. The next best index is the services per pregnancy. Since such measures could include a deleterious management factor (excessive breedings prior to ovulation), one should also determine the estrous periods per foaling (or pregnancy). These figures should be calculated for each of the 3 mare groupings. In addition, and particularly when the apparent fertility measures are low, the management and veterinary programs under which he has performed should be evaluated. This includes the nutrition and parasite control program of the mares (and stallion), use of artificial lights, teasing and palpation program as well as immunization, diagnostic and therapeutic procedures, particularly as they relate to the genital system. An attempt should be made to obtain a history of his offspring with particular emphasis on possible congenital defects.

Try to obtain a complete history of the stallion's past diseases
and vaccinations as well as any medications including those received as a youngsters. In the case of race and performance horses it is often true they have received medication. It is desirable to know as much as possible about the drugs, dosage and frequency of administration, although such information can be difficult to obtain.

4. General Physical Examination:

Once this history and identification are recorded, the horse is given a general physical examination. It is essential that in order for a stallion to be considered fertile it must have the desire and ability to deliver fertile sperm to the cervix or uterus in an estral mare. Any defects which detract from this necessary ability are recorded. Specifically, the horse should not be blind or severely lame or have faulty conformation that interferes with mounting, intromission or thrusting.

After completing the general physical examination, a blood sample should be taken and checked for evidence of Equine Infectious Anemia (EIA) unless this has been done in the last 3 months. (See page 42, paragraph 2)

5. Artificial Vagina:

For semen of the horse to exhibit its maximum quality, it should be collected with an artificial vagina (AV). Other methods of collection, such as the condom, do not provide as reliable or suitable a sample. There are currently three types of closed artificial vaginas available in the U.S. All are satisfactory and the examiner should select one and learn its idiosyncrasies. The Nishikawa model provides good pressure on

a,b,c - see Appendix 3
the glans, which may be unnecessary, but also provides the collector with a shower of water unless the air vent is fitted with a blow-off tube or the cap is covered with a balloon. The Missouri model is more adaptable in that both air and water can be added. Furthermore, selective manual pressure can be applied to the glans. The Colorado model retains heat very well but is heavier than the other two. It is wise to keep extra, non-used reserve liners on hand under refrigeration if possible until put into use.

There are no commercially open-ended AV's but they can be constructed with tubing available in hardware stores or laboratory supply houses. The advantage of the open-ended AV is that it permits the use of the urethral prosthetic device which enables the collection of gel-free, bacteria-free semen for use in selected cases. Some horses require more training to this AV than to a closed one.

The rubber liner that comes in contact with the penis and semen must receive careful attention. Because residues of soap, detergent or disinfectant can have detrimental effects on sperm, the liner is washed in hot water, minimum 3 times, with non-residual soap or detergent, then rinsed several times in hot running water and then immersed in 70% alcohol for at least 20 minutes. If the liner is to be reused promptly it should be rinsed liberally with physiological saline (PSS) before use. If not used promptly, it is hung in a dust free cabinet to dry. When dry, powder with sterile talcum powder. Upon removal for use it should be rinsed with PSS a minimum of 3 times to remove all traces of powder.
In preparing for collection, the liner is filled with 50°C (121°F) water to reach a final temperature of 45°C (113°F). A few stallions prefer a final temperature above 45°C which necessitates the filling water be above 50°C. Depending on which AV is used, this can be a questionable practice first shown by Cooper[18], subsequently confirmed,[34] who found permanent damage to motility and respiration of sperm above 45°C (112°F) which accelerated above 48°C (118°F).

The high temperature is not a problem with the Missouri AV because the glans and urethra are beyond the heated portion. It is only a slight problem with the Nishikawa AV and a serious problem with the Colorado AV where the semen is in direct contact with the hot liner.

After the jacket is filled with water of the proper temperature and before closing the port, when present, sterile surgical jelly which contains no disinfectant(d) is applied as lubricant to the interior of the liner using a glass rod or else an arm covered with a sterile plastic sleeve which is inserted into the vagina. This forces out the excess water and simultaneously lubricates the liner.

Either before or after filling the liner, a receptacle is attached to the AV to collect the semen. A variety of semen receptacles have been used. Although the plastic baby bottle has been widely used, the Whirl-Pak bag(e) has the advantages of being sterile, disposable and non-adherent for sperm. The plastic baby milk bottle has the draw-back that it must be washed until chemically clean and sterilized between each use.

d, e, - see Appendix 3
A recent innovation to the baby bottle is the development of plastic inserts for the bottle - so called "disposable bottles"(f,g) and an AV adapter for the Missouri model.(h) To facilitate attachment of the Whirl-Pak bag to the liner of the Missouri AV a Teflon fitting(47) has been designed (i) or it can be taped on. In cold weather when collecting in a non-heated area, the semen receptable should be protected by enclosing it in a second, warmed bag fashioned from a space blanket. An additional plastic bag filled with warm water may be added to this protective cover. (The Colorado model has the advantage of being able to maintain sufficient warmth under conditions of severe cold and draft.) Although stallion sperm seem less susceptible to cold shock than those of the bull and ram, they are permanently damaged by exposure for 10 minutes to temperatures below 20°C (68°F) as well as above 45°C (112°F).(18)

6. Teasing, Microbiological Examination and Preparation for Semen Collection.

a. Preparation for Collection:

In the past fertility evaluations have been made, by collecting 2 ejaculates 1 hour apart, after 1 week of sexual rest.(63) The difference in sperm numbers between the 2 ejaculates provides a measure of epididymal sperm reserves. On the basis of work of Gebauer and his co-workers,(25, 27) it appears that in order to make a reliable estimate of daily sperm output (DSO) it is necessary that 2 ejaculations be collected.

f, g, h, i, - see Appendix 3
and evaluated 1 hour apart after 1 week of twice daily collections rather than a week of sexual rest.

If it is not possible to take a stallion down to DSO we recommend that, during the breeding season, the horse be evaluated while in his usual collection or usage frequency. During the non-breeding season, 2 collections should be made after 1 week of sexual rest. It must be realized that frequency of usage may significantly affect semen quality in stallions.

b. Teasing:

Following the physical examination, the horse is taken to an estral mare in a teasing chute where the stallion's behavior is noted and recorded. It is desirable that the horse show intense interest in the mare as a sign of normal, healthy libido. He should show the normal behavior of becoming restless, pawing and usually whinnying. He will approach the mare, usually near the neck, from where he works down her side nudging and nipping. The nuzzling and smelling continues around the vulva occasionally followed by the "Flehman" reaction. Erection is usually present shortly after sighting the mare but may occur anytime during teasing. If erection fails to occur, he is examined for presence of a stallion ring or anatomical problem.

c. Washing Stallion:

With the horse still in view of the mare, the penis, (Appendix 4a) prepuce, fossa glandis and its diverticulum (Appendix b) are thoroughly washed and rinsed with warm water, non-residue soap (such as Ivory soap), not a detergent, and absorbent cotton or disposable cloth or wet strength paper towel until dirt and sebum is no longer picked up.
A thorough examination of penis and sheath are made during the washing procedure paying attention to the landmarks in the diagrams in Appendix 4a. The pails used for washing and rinsing should be separate, clean and of stainless steel or plastic. Lining pails with fresh, disposable, plastic bags each time is highly recommended. The glans is sponged to dryness after the last rinse.

**d. Microbiological Examination:**

The examiner should swab the urethra in a sterile manner after the penis has been washed and dried. The glans should be rubbed briskly with a damp fresh towel or cotton to initiate flow of pre-seminal urethral fluid that will cleanse the urethral lumen and reduce likelihood of swab contamination. The swabs are promptly streaked on plates (slants have too small a surface) of blood agar or other appropriate media for isolation of aerobic bacteria.

If there is a history of or a suspicion of a Pseudomonas problem with the horse it is advisable to examine the unwashed, erect penis under ultraviolet light in darkness or subdued light and collect swabs from the areas which exhibit specific fluorescence. (43)

See Appendix 18 for Code of Practice for handling the possibility of Contagious Equine Metritis.

7. **Semen Collection:**

The estral mount mare is prepared by having her tail wrapped with fresh gauze bandage or other hygienic wrap such as a plastic sleeve. This is done so tail hairs can be readily controlled and kept out of the field of operation. The mare is restrained in a dust-free area with good footing.
for her as well as a good approach and footing for the stallion. The person handling the stallion then allows it to approach and mount the mare in a controlled manner. Control is obtained with a chain shank over the nose, upper gum or in the mouth or with a bit.

As the horse mounts the mare, the collector directs the penis away from the mare while permitting urethral secretions (pre-sperm fraction) to rinse the urethra as they are voided and not collected. When these secretions diminish, and when and if possible, the interior of the urethra is wiped in a sterile manner with a sterile swab for subsequent detection of bacteria. With very vigorous stallions swabbing will have to be done after the wash and before mounting.

Intromission into the AV is facilitated and the horse allowed to ejaculate while the collector places the index finger against the ventral shaft of the penis so the urethral pulsations, which indicate possible ejaculation, can be felt and counted (see B. - 3b. Behavioral Problems). Following ejaculation the collector tilts the AV so that the semen drains into the sterile semen container. Upon dismount, the urethra is again swabbed.

The manner in which the horse approaches and mounts, intromits, and ejaculates is noted and deviations from normal are recorded.

Occasional stallions, particularly draft breeds, refuse to ejaculate into an AV (see B. - 3b. Behavioral Problems). One must make certain that all conditions are optimal. Particularly important are the temperature and pressure in the AV. Patient training is in order or, as a last resort,
condoms (k) can be used. Condoms should be avoided because they cause more organisms to be included in the semen from the shaft and glans and make microbiological interpretation very difficult.

The examiner then performs those tests on the semen which must be done immediately (i.e. initial motility, pH). The external and internal genital organs are then given a thorough physical examination. A second ejaculate is collected one hour after the first.

8. **Semen Examination:**

Stallion sperm are deleteriously affected by oxygen, sunlight and drastic changes in temperature. Therefore, do not shake semen samples as this exposes them to more oxygen, keep out of light and in the dark, keep them at a uniform temperature and fill the container to exclude air.

The evaluation of semen is divided into three sections:

1) Those tests done promptly after collection; 2) Those done soon but need not be done promptly; 3) Additional tests that require more time and equipment. Although many of the latter tests have not been extensively investigated, they have been very beneficial, on occasion, in detecting a cause of infertility when other tests have not helped. The potential value of these tests should not be underestimated.

The various semen tests are conducted on gel-free semen. A distinct advantage of the Whirl-Pak bag and disposable baby "bottles" is that in holding them up to the light one can readily see the gel, since it

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k - see Appendix 3
floats on top. By touching the surface of the gel with the 20-50 cc syringe tip, gently aspirating and then raising the syringe above the surface while continuing to aspirate one can remove all the gel neatly and cleanly. The gel removes a highly variable % of the sperm of the ejaculate. An alternative to aspiration of the gel is to incorporate a filter in the AV to remove the gel. This is not only much less efficient for gel removal but the filter removes up to 25% of the sperm of an ejaculate before counting. The longer the gel sits in the filter the more goes through. Aspiration removes only the sperm contained and adherent to gel. Furthermore, many ejaculates do not contain gel particularly during the non-physiological breeding season.

a. Tests To Be Done Immediately:

1. Gross Evaluation:

The volume of both the gel and gel-free portions are recorded. The color (milky white, gray, pink, red), degree of opacity (clear, opaque) and consistency (watery, thick) are all recorded. All materials the semen contacts should be sterile, chemically clean and at body temperature. The semen is poured through a sterile in-line milk filter into a sterile, disposable plastic beaker. This step is less important if the penis has been washed properly and the gel has been completely aspirated. Any gross abnormalities, such as presence of blood, urine and pus, as well as the volume are recorded. An aliquot of well-mixed, gel free, filtered semen is taken for subsequent determination of

1, m - see Appendix 3
sperm concentration,

Because of the watery nature of the gel-free portion of stallion semen, sperm will sediment rapidly. It is therefore important to always gently but thoroughly mix the semen before taking a sub-sample. Mix by rotation, not by shaking, which produces a persistent foam and adds air, the oxygen of which, can be detrimental to sperm.

ii. Sperm Motility:

A drop of mixed, raw semen from a disposable Pasteur pipette or wooden stick is placed on a warm (37°C) slide and covered with a prewarmed cover slip and examined microscopically. To keep slides, cover slips, beakers, syringes, filters, bags, pipettes, sticks warm one can use a small incubator or warming tray. Initial motility of sperm is most reliably estimated in the raw state rather than in extender, as has been recommended, since many extenders have the ability to increase both the vigor and percentage of motile sperm. Agglutination is rarely a problem if examination is made promptly.

Motility is classified as circling, oscillating, serpentine or progressive. The most important and critical aspect of motility is progressive motility - that is, the cells are actively moving forward. Large, circular motion of some normal sperm, is due to the high incidence of normal, abaxial connections between sperm head and neck. Record both the total (T) percentage that are motile as well as the percentage that are

n, o, p, q - see Appendix 3
progressively motile (P) i.e. (T/P). The presence of non-sperm elements such as spermatids, spermatocytes, erythrocytes, leukocytes, epithelial cells, crystals, and foreign matter are noted and quantitated. For technique of motility estimation see Appendix 17.

iii. pH:

Next the pH is measured, preferably with a standardized pH meter. Only as a last resort should short range pH paper be used. A pH over 7.5 in first ejaculates can be normal but is suggestive of infection or contamination with alkaline material such as soap or urine.

b. Subsequent Semen Tests:

i. Semen Morphology:

Semen morphology can be evaluated in the liquid, preserved (33*) state (buffered formal saline - BFS) under phase contrast or phase interference microscopy, in dry, fixed and stained samples (Williams(89) and Cassaretts(14) stains or with background stained samples (India ink* and eosin-nigrosin*) - the latter is available from the Society. To facilitate the development of uniformity and accuracy, it is recommended that when possible morphological examination be made with BFS preserved samples and when not possible the examination be made with eosin-nigrosin.

The advantage of BFS preserved sperm under phase microscopy is that it is probably the most reliable light microscopy technique for evaluating acrosomes and midpieces. If placed in tightly sealed

* see Appendices 5, 8, 9.
rs - see Appendix 3
The sample is good indefinitely. It is suitable for other abnormalities as well, however, it is unsatisfactory for evaluating somatic cells. The cells can be smeared on a glass slide from the sediment and then stained with Giemsa or Wrights. Eosin-nigrosin and India ink are probably a very distant second best for evaluating acrosomal abnormalities. They are quite suitable for evaluating other sperm abnormalities although adherent gel droplets can create confusion concerning the shape of the midpiece and the presence of droplets; it is therefore essential that the gel be removed beforehand. India ink as well as eosin-nigrosin are useless for somatic cells. The advantage of these "stains" is that there is no preparation involved, other than smearing, and samples can be evaluated immediately.

The Williams and Casarett's stains both produce high quality positive stains of the spermatozoa. They are not suitable for somatic cells. There is some evidence that Casarett's stain can produce artifactual changes. (2)

The eosin-nigrosin is frequently referred to as a live-dead stain. Because of variations in environmental humidity it is too capricious in most areas to rely on the live-dead aspect. It provides a useful background stain but it is less satisfactory than BFS for acrosomal and somatic cell evaluation.

For leukocyte evaluation a blood stain such as
Wrights * or Giemsa * is best. Some of the newer, quick, one step stains are adequate.

In making semen smears for staining, the sperm density in the smear must be adjusted so as to be thin enough to prevent overlap of sperm. Samples that are too dense for making smears for air drying are diluted with normal saline. Four additional slides are prepared but left unstained until it is determined whether a Wright's or other stain is needed for a differential leukocyte or spermatocyte count.

The morphological abnormalities of sperm are categorized along the lines depicted by Bielanski (4) and recorded (see Appendix 1 and 2). A method of categorization is in Appendix 12.

ii. Sperm Count:

Sperm concentration can be determined by hemocytometer \( t \) count (see Appendix 10) or by spectrophotometer (see Appendix 11). The least expensive, reliable counting is done with a hemocytometer coupled with one of the reliable pipetting systems. Unless one does numerous sperm counts, the spectrophotometer is too much of an investment. Furthermore when sperm numbers are very low or many non-sperm cells or debris are present, one must resort to the hemocytometer. The non-sperm materials disrupt the light path and will be counted as sperm. We have seen stallions with no sperm in the ejaculate, only spermatocytes and spermatids, yet were thought to have sperm because of the response of the

* see Appendices 8,9
\( t,u \) - see Appendix 3
spectrophotometer. Spectrophotometric counting of stallion sperm was introduced by Haag in 1959 by adapting techniques then in use for bull semen. Colorado workers subsequently demonstrated that a wavelength of 525 μm was superior to that of 625 μm used for bull sperm.

\[
\text{Total sperm per ejaculate} = \frac{\text{volume of gel-free semen in cc's}}{\text{X per cc}} \times \text{number of sperm}
\]

\( ^{32} \)

**c. Additional Tests**

**i. Longevity of Motility in Raw Semen:**

For estimating the longevity of motility an aliquot of well-mixed semen is then used to fill a warm, 5 ml sterile tube which is kept in a draft-free, dark environment at 72°F (22°C). The tube should be filled to exclude air because of detrimental effect of oxygen on motility. The semen is mixed and an aliquot examined every hour until 10% or less of the sperm remain progressively motile. It is inadvisable to use semen to which extender has been added for estimating initial motility as the extender frequently artificially enhances sperm motility and may prevent clumping.

**ii. Longevity of Motility in Extenders:**

Testing the longevity of sperm in at least one acceptable extender may add additional information concerning the semen quality of a horse or in assisting in the management of stallions of known infertility.

An aliquot of semen is mixed with equal parts of extender and examined as described for raw semen.

\( ^{v} \) see Appendix 3

\( ^{*} \) see Appendix 19
iii. Washing Sperm:
A technique for washing sperm has been developed. It is used in cases in which poor motility is the predominant finding in horses of low or suspected low fertility. Sperm are washed by adding equal parts of extender to a well-mixed aliquot of semen. Appendix 16.
The mixture is centrifuged for three minutes at 300 G's (1500 RPM with 7.5 cm arm), the supernatant is carefully poured off or aspirated, and the soft pellet of sperm is resuspended in extender equal to the original volume of the semen sample and is ready for estimating longevity of motility.

iv. Agglutination:
Record whether or not agglutination (clumping) of sperm occurs on initial examination of raw semen. If it occurs, record whether the agglutination is head to head, or tail to tail, as well as the degree to which it occurs. A slight degree involves two or three sperm per clump. When of moderate severity sperm will develop star-shaped formations. If clumps are larger than this, it is considered severe.

v. Microbiology:
A sample should be taken from the sterile semen collection device (Whirl-Pak or baby bottle) promptly for culture of organisms providing the semen has been collected in a clean, sterile manner and in a sterile receptacle.

9. Physical Examination of Genital Organs:
The physical examination of the genital organs is most readily

w - see Appendix 3
performed after ejaculation as the horse will be more tractable. An additional advantage of palpation between ejaculates is that if there are inflammatory changes in the pelvic organs (prostate, seminal vesicles, ampullae) the leukocyte count in the second ejaculate will tend to be greater than in the first if there has been a vigorous intervening massage. The penis and sheath will have been examined when the penis was washed. (Appendix 4) The scrotum and testes are palpated for their presence, size, shape, texture and location. Testes are measured in three dimensions by using the index finger and thumb as a caliper or a metal caliper can be used. For the purpose of standardization, use of the calipers is preferred. The measurements should be repeated three times to assure their accuracy and then recorded. Each of the three divisions of the epididymis is palpated throughout for size, location and texture. The head of the epididymis is cranio-dorsal, the body is dorso-lateral while the tail is causal to the testicle. (Appendix 13) Deviations for normal are usually due to anomalies or torsion. In addition, the size and location of the ligament of the epididymis (gubernaculum) is noted. It is usually less than one cm long and 0.5 cm in diameter. It is located dorsal and posterior to the tail of the epididymis and along with the tail of the epididymis is an aid in detecting testicular torsion.

The examination of external genitalia is completed by following the spermatic cord to the external inguinal ring (which is also palpated for abnormalities).

- see Appendix 3
The internal genital examination involves palpation, per rectum, of the isthmus of the prostate which is a firm 2 x 4 cm structure about wrist depth, cranial to the anal sphincter. (Appendix 14) The seminal vesicles and ampullae are then palpated. The seminal vesicles are located lateral and slightly cranial to the prostate. The ampullae can be located on the floor of the pelvis directly anterior to the prostate. The ampullae can be followed anteriorly along the floor of the pelvis. It is common for the inexperienced to fail to detect empty seminal vesicles because of their soft, pliable nature. The empty seminal vesicles are thin walled, vesicular structures about 2 to 7 cm long and 2 to 3 cm in diameter. The vesicles are usually more prominent after the horse has been vigorously teased. At this time they will be distended with fluid and can be readily identified. The ampullae are more readily detected and palpated than the empty seminal vesicles. The ampullae are soft, glandular structures about 10 to 20 cm long and 0.7 to 2 cm in diameter. (Dependent on age) The ductus deferens, which is about 0.6 cm in diameter, passes through the internal ring and becomes the ampullae. (See Appendices 14 and 15) The size and consistency of the vesicular glands and ampullae are noted and recorded.

The two internal inguinal rings lie 10 cm cranio-ventral to the pelvic inlet and just lateral to the midline. Location is achieved by folding one's fingers against the palm of the hand and then unfolding the fingers while pushing the finger tips against the abdominal wall over the region of the internal inguinal ring. Once this is located, the region is checked for adhesions and hernias. The ductus deferens and the pulse
in the spermatic artery are palpated.

10. **Fiberscopic Examination of Penile & Pelvic Urethra**

Fiberscopic examination of the penile and pelvic urethra is occasionally a very useful adjunct to physical examination of the penis and internal genital organs. This examination is best performed in the presence of an estral mare with the stallion tranquilized with a non-phenothiazine tranquilizer. The penis is then washed and rinsed 3 times with disinfectant soap. The sterilized scope is then lubricated with a water-soluble lubricant and carefully passed up the urethra by one operator wearing sterile rubber gloves. A second operator observes the urethra, looking for abnormalities, as the scope is passed. The pelvic urethra has the openings of the various glands as well as the colliculus seminalis with 2 orifices of the ampullae and 2 of the seminal vesicles. (See diagram, Appendix 15)

B. **INTERPRETATION OF INDIVIDUAL PARAMETERS**

1. **Stallion Identification:**

   It is essential that after passing judgement on the potential fertility of a stallion the examiner be able to identify the stallion in question at a later date. Therefore, it is wise to photograph as well as to identify each stallion by written description of major features and tattoo.

2. **Congenital Defects (Genetic or Possibly Genetic):**

   There are very few proven congenital defects which are known to have either a deleterious effect on fertility or are obviously detrimental.

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y - see Appendix 3
a. Cryptorchidism is generally considered to be genetically controlled in all species. The evidence that it is genetically controlled in the horse is inconclusive. Nonetheless, cryptorchidism constitutes a particularly damaging and undesirable defect which, when bilateral, sterilizes the animal. Uni- or bilateral cryptorchid stallions are considered to be UNSATISFACTORY PROSPECTIVE BREEDERS.

b. Combined Immunodeficiency (CID) is known to be a recessive defect restricted to Arab horses thus being transmitted by both sire and dam (71).

Unfortunately there is not yet a test for detecting the carrier state (61). This means that all 3 criteria for establishing a firm diagnosis in the foal must be present. They are (57):

1. Lymphopenia of less than 1000/mm³
2. Absence of IgM
3. Hypoplasia of the spleen, thymus and lymph nodes

When it is documented that a stallion has sired a foal with CID, that stallion should be considered an UNSATISFACTORY PROSPECTIVE BREEDER.

c. Parrot Mouth: This is a malformation caused by upper jaw overgrowth or a short lower jaw. It has a genetic cause, possibly dominant (41).

d. Hemophilia: This rare disease is characterized by the appearance of hematomas over various parts of the body, especially around joints and body prominences in young foals. There is a prolonged clotting time and deficiency of anti-hemoglobin globulin. It is postulated to be a sex-linked recessive trait (41).

e. Complete Mature Cataracts: There is opacity of the entire lens
at birth. Usually bilateral and causes blindness.\(^{41}\)

\(f.\) **Aniridia:** Bilateral absence of iris. Genetic and probably an autosomal dominant.\(^{41}\)

\(g.\) **Wobbler:** Ataxia of young horses, especially of rear legs. Characterized by sudden or insidious onset with subluxation of cervical vertebrae.\(^{41}\)

\(h.\) **Multiple Exostoses:** Numerous bony prominences with distinct tendency to be bilaterally symmetrical on or near costochondral junctions, as well as most long bones and pelvis. Condition is genetic in origin and thought to be dominant.\(^{41}\)

3. **Nongenital Defects:**

Nongenital defects which have an effect on breeding ability are usually detected during the general physical examination or during the breeding act.

a. **Physical Defects:**

Serious eye defects, including partial blindness, can interfere with the ability of the stallion to mount and stay mounted. Other non-generative problems having a bearing on breeding ability which can be encountered are: 1) hind leg weakness due to damage to the spinal cord as a result of trauma, 2) wobbler syndrome,\(^{73}\) 3) spinal cord myelitis,\(^{73}\) 4) neuritis of the cauda equina,\(^{73}\) 5) severe laminitis and 6) viral myelitis due to equine rhinopneumonitis. Sources of pain in the hind limbs including, but not limited to, osteoarthritis of the hip, stifle, hock or digital joints may not be as obvious but can have their effect in that the
horse will make several mounts before ejaculating. (48 )

b. Behavioral Problems:

Another problem area encountered early in fertility evaluation of stallions is behavioral. This is particularly true in instances of inexperienced stallions and racing stallions being considered for retirement to the breeding farm. During their racing career they are often punished for sexual responses. As a consequence, upon presentation to an estral mare, they may exhibit difficulty in expressing normal behavior because of the prolonged suppression. The normal horse should be anxious and able to mount, to seek and find the vulva or be willing to insert in the AV, to thrust and to promptly ejaculate. Interest in the estral mare can vary from normal to complete disinterest, apparent aversion, or even fright. Some inhibited stallions will fail to obtain an erection in the presence of a mare and may be more interested in feed, yet when left alone in a box stall will masturbate. Other forms of abnormal behavior include greatly prolonged reaction time, mounting without erection, multiple mounts before intromission, multiple insertions before ejaculation and abusing the mare. The collector is alerted to the lack of ejaculation when he is unable to feel the normal three to nine urethral pulsations as well as by the lack of semen on dismount. Some stallions will occasionally "flag" their tail without ejaculation. Less often pulsations without ejaculation occur.

Treatment of these stallions requires patient retraining of the horse for breeding. (64 ) This is accomplished by presenting a variety
of estral mares to the horse for at least fifteen minutes twice a day in quiet, undisturbed surroundings until normal behavior patterns are established. Some older mares are useful for this purpose, particularly if the two can be turned into an isolated paddock together. The length of time it takes to attain normal breeding behavior may take only a day or several weeks. The wide variation is probably dependent on the magnitude and duration of the altered behavior as well as their innate sex drive.

The apparent diminished frequency with which stallion rings are used is probably related to the fact that there is no documented evidence that masturbation has a detrimental effect on performance or even that ejaculation usually occurs during masturbation.

When abnormal steroid levels are associated with behavioral abnormalities serious ethical questions arise as to the handling of such problems. For instance, it is possible to raise low androgen levels directly by administration of exogenous testosterone or indirectly by luteinizing hormone. The elevated levels do not necessarily facilitate the attainment of erection and ejaculation. However, the really serious question to be answered involves the causation of the low levels - are they the result of a genetic defect or of some acquired condition? In the first instance, the animal should be considered an unsatisfactory candidate for breeding purposes, in the second instance some form of therapy may be morally justified. It is usually not possible to prove the genetic basis of such problems. Therefore, the only alternative is to not pass the animal with abnormal steroid levels coupled with abnormal behavior. That does not mean it is sterile and if owner wishes to risk his
time and money many horses that do not fill these criteria will get mares pregnant.

4. Genital Physical Examination:

In order to conduct a physical examination of the genital organs, one must be familiar with the normal location, size and texture of each organ as well as their abnormalities,

a. Scrotum:

The scrotum is rarely the site of a disease process and, when so, it is usually of traumatic origin. In addition, edema of the scrotum can reflect systemic illness such as equine infectious anemia\(^{(16)}\) and equine viral arthritis\(^{(12)}\) or topical application such as an insecticide spray. Edema can cause transient inhibition of spermatogenesis by interfering with heat exchange. Neoplasia, scrotal dermatitis, and other infectious problems are rare.

b. Penis and Sheath:

The penis and sheath are subject to more frequent traumatic injuries than the scrotum and should be examined closely. In addition, the penis should be carefully examined for evidence of anomalies of the shaft\(^{(23)}\) and of the urethral process. (See diagram in Appendix 4b)

The examiner should also be familiar with the penile changes associated with coital exanthema (equine herpes) virus\(^{(28)}\), summer sores (habronemiasis)\(^{(44)}\), sarcoid,\(^{(44,59)}\) melanomas, and squamous cell carcinoma\(^{(44)}\) and warts.\(^{(59)}\) A stallion with these lesions should not pass the examination for fertility. With the first four conditions, a re-examination should be performed as soon as the conditions are cleared up.
c. Testicles and Epididymides:

i. Size and Location:

The normal stallion has two scrotal testicles which lie with their longitudinal axis horizontal. In the normal mature, Standardbred and Thoroughbred, each testicle is 8 to 10 cm long by 6 to 7 cm high by 5 cm wide. The head of the epididymis lies on the anterior, dorsal aspect of the testicle where it passes from medial to lateral around the attachment of the spermatic cord. The epididymis continues posteriorly on the dorso-lateral aspect of the testis. The head (3 x 4 x 1 cm) and body (1.5 cm diameter x 11 cm) of the epididymis are palpable but these organs are difficult to outline precisely. The tail of the epididymis lies at the caudal pole of the testicle. Its normal size in the adult Standardbred and Thoroughbred is variable but about 3 cm x 2.5 cm diameter. (See Appendix 13)

The tail of the epididymis and the gubernaculum serve as excellent landmarks for identifying torsion of the spermatic cord.

ii. Epididymal Problems:

Few diseases of the stallion epididymis are known.

Occlusion of the epididymal duct as a result of spermatic granulomas developing in vestigal, blind tubules has been observed. (50) An abscess-like defect has been observed in the tail of the epididymis in association with repeated cultures of Streptococcus zooepidemicus. (81)

iii. Scrotal Width:

It has been shown that there is a direct correlation between testicular width, grams of testicular parenchyma and sperm
production. (25, 27, 80)

**EFFECT OF AGE ON TESTICULAR SIZE IN STALLIONS**

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>2-3</th>
<th>4-6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of stallions</td>
<td>11</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>Measurement (mm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scrotal width</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>96ª</td>
<td>100ª</td>
<td>109ª</td>
</tr>
<tr>
<td>2 s.d. (range)</td>
<td>81-111</td>
<td>85-115</td>
<td>95-124</td>
</tr>
<tr>
<td>Left width</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>55ª</td>
<td>57ª</td>
<td>61ª</td>
</tr>
<tr>
<td>2 s.d. (range)</td>
<td>46-63</td>
<td>49-66</td>
<td>52-70</td>
</tr>
<tr>
<td>Right width</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>53ª</td>
<td>55ª</td>
<td>60ª</td>
</tr>
<tr>
<td>2 s.d. (range)</td>
<td>43-62</td>
<td>45-65</td>
<td>50-69</td>
</tr>
</tbody>
</table>

Means with no common superscripts are different (P < 0.05)

In the next table are the testicular widths of Standardbred stallions by the age and whether they were on a breeding farm or racetrack. (91)

It is shown that both training and racing have an adverse effect on testicular size compared to being on a breeding farm. It was also shown that "medication" had a further adverse effect on testicular size. (91)

It is therefore clear that testicular width is correlated with testicular parenchyma which increased with age up to a certain limit which, in turn, is correlated with sperm producing capacity. (25, 80) This capacity may be reduced by training, racing and certain medications including, and particularly, those with androgenic activity. Depending on a variety of factors this atrophy may be transient or permanent. (79)

Thus, testicular and scrotal width can be utilized in the examination for estimating sperm producing potential of each testicle. In so doing, one must also estimate the texture and shape of the testicle as a means toward evaluating the normalcy of the parenchyma.
iv. Spermatic Cord:

The cords should be of equal size and uniform diameter (about 2.5 cm). Varicosities of the stallion pampiniform plexus are uncommon but have been observed. (85)

On an annual basis torsion of the spermatic cord is seen in about 0.1-0.5% of stallions presented for fertility evaluation at the University of Pennsylvania's fertility clinic (Hofmann Center). It appears to be more common than is represented in the literature. (36,60,77)

This is probably due to the fact that it is usually clinically silent and appears to be innocuous.

<table>
<thead>
<tr>
<th>Age</th>
<th>N</th>
<th>Left X ± SE</th>
<th>Right X ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>256</td>
<td>4.9 ± .04</td>
<td>5.2 ± .04</td>
</tr>
<tr>
<td>3</td>
<td>178</td>
<td>5.1 ± .06</td>
<td>5.4 ± .06</td>
</tr>
<tr>
<td>4</td>
<td>84</td>
<td>5.1 ± .09</td>
<td>5.3 ± .09</td>
</tr>
<tr>
<td>Racetrack Breeding Farm</td>
<td>12</td>
<td>5.2 ± .86</td>
<td>5.7 ± .62</td>
</tr>
<tr>
<td>5</td>
<td>66</td>
<td>5.1a ± .11</td>
<td>5.2a ± .10</td>
</tr>
<tr>
<td>Racetrack Breeding Farm</td>
<td>13</td>
<td>5.7b ± .27</td>
<td>6.1b ± .18</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>5.1 ± .15</td>
<td>5.4a ± .13</td>
</tr>
<tr>
<td>Racetrack Breeding Farm</td>
<td>12</td>
<td>5.5 ± .22</td>
<td>6.1b ± .20</td>
</tr>
<tr>
<td>7</td>
<td>33</td>
<td>5.2 ± .14</td>
<td>5.4a ± .12</td>
</tr>
<tr>
<td>Racetrack Breeding Farm</td>
<td>16</td>
<td>5.6 ± .24</td>
<td>6.3b ± .19</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>5.2 ± .17</td>
<td>5.2a ± .21</td>
</tr>
<tr>
<td>Racetrack Breeding Farm</td>
<td>14</td>
<td>5.7 ± .22</td>
<td>6.3b ± .29</td>
</tr>
<tr>
<td>9-13 Racetrack Breeding Farm</td>
<td>25</td>
<td>5.3a ± .15</td>
<td>5.5a ± .14</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>5.8b ± .15</td>
<td>6.3b ± .13</td>
</tr>
</tbody>
</table>

a,b = within an age group for each side, means with different superscript letters are different (P < 0.05)
Transient testicular torsion occurs more frequently than the permanent form. In the transient form the testicle can be returned to its normal position by manual manipulation and is occasionally found in that position spontaneously. The effect of the transient torsion on spermatogenesis is not known but many stallions with this affliction produce acceptable numbers of spermatozoa. Permanent torsion of the testis may have deleterious vascular consequences which suppress spermatogenesis or even cause clinical signs when more than 180°.

v. Testicle:

The testicle, with its attached epididymis, should be freely movable within the cavity of the tunica vaginalis. Inability to do this indicates the probable presence of adhesions. Because the scrotal skin is soft and pliable it is wise to observe this skin while performing the manipulations since adhesions can pull the skin along without being detected by palpation alone. The best time to palpate the testes is after ejaculation when the scrotum is relaxed, and the testes are less apt to be reflexly withdrawn. Traumatic injuries can result in adhesions, hematocele, and even intratesticular hemorrhage, all of which may have an adverse effect on spermatogenesis.

Relatively minor degrees of variation in tenseness or turgidity of the testicle may result from the contraction or relaxation of the smooth muscle of the tunica albuginina. This variation can occur from minute to minute but is usually uniform throughout each organ. Except for this variation, the testicle should be smooth and of uniform texture throughout except for the slight undulations produced by the numerous
veins of the tunic.

vi. Testicular Neoplasms and Abscesses:

Irregularities larger than those noted above as well as irregularities in form or texture should be considered pathological. Neoplasms appear to be infrequent and are essentially limited to seminomas, teratomas, and lipomas. Abscesses appear to be rare occurrences as are varicosities, hematocoeles and intratesticular hemorrhage. Migratory parasites have not been known to produce clinically detectable changes.

vii. Cryptorchidism:

Cryptorchidism is common in stallions but unfortunately the genetic control, if any, is not known. Congenital cryptorchidism renders a stallion an UNSATISFACTORY PROSPECTIVE BREEDER.

viii. Testicular Hypoplasia:

Testicles that are small and uniform in shape and texture present a serious diagnostic challenge since they may either be hypoplastic or have undergone degenerative atrophy. Differentiation of these two conditions is crucial to the proper disposition of the horse since the implications are completely different. Testicular hypoplasia is congenital and probably genetically based, whereas testicular degeneration is an acquired condition.

Hypoplastic testicles are those which failed to undergo normal testicular development. Such testicles can vary from those that are of normal texture and only slightly smaller than normal to those that are firm and considerably smaller than normal.
Testicles suffering from degenerative atrophy have first undergone normal development and have then atrophied as a result of degeneration. Both conditions can be unilateral or bilateral and can vary in degree from slight to severe. They both result in decreased sperm production. These two conditions are differentiated on the basis of historical evidence since there are not seminal, anatomical or histological criteria by which they can be dependably separated.

In four cases of suspected hypoplasia, it was not possible to detect chromosomal abnormalities using current methods. (52)

Since hypoplasia is developmental in origin it can arise either postnatally (and prepuberally) or prenatally. Whether of either prenatal or congenital origin it can result from noxious influences on the fetal gonad or be genetic in nature.

The historical evidence that is necessary for differentiating hypoplasia from atrophy is that the undersized testes either did develop to a normal size and produce sperm in normal numbers, or they did not. In the first instance they would now be atrophic while in the latter instance they would be hypoplastic. In many cases there is insufficient evidence to determine the course by which the testicles reached their present subnormal size. Nonetheless, such stallions are UNSATISFACTORY PROSPECTIVE BREEDERS when they produce insufficient morphologically, normal, progressively motile sperm. (see C 6)

ix. **Testicular Degenerative Atrophy:**

Degenerative atrophy is an acquired condition which is the final path of a myriad of causes. However, the effect of most of
these agents has not been documented in stallions.\(^{(29)}\) In spite of knowing of the many agents capable of inducing atrophy, it is seldom possible to determine the precise cause in any given case. Certainly one must consider infectious agents as having the potential of causing orchitis with subsequent permanent degenerative atrophy.\(^{(42)}\) The role various equine viruses have to play in producing this condition is uncertain.

In addition to the suppressive effect on spermatogenesis of the various gonadal steroids, such as testosterone, estrogens and progesterone, there is increasing concern about the potential effect of the anabolic steroids on testicular function. It is far from clear what their potential for permanent damage is,\(^{(3,11,13,78,91)}\) Whether or not steroid produce a permanent effect may be largely dependent on the amount and duration of administration as well as age at administration. The increasing popularity of these newer agents in racing stallions must give cause for concern until more is known about their potential effects on future reproductive performance.\(^{(91)}\)

5. **Semen Examination:**

   a. **Representativeness:**

   Two ejaculates are collected an hour apart so the four criteria of representativeness\(^{(63)}\) can be applied.

   These are: 1) the volume of both ejaculates should be about the same, 2) the total number of sperm in the second ejaculate should be about half the first, 3) the pH should stay the same or go up slightly, 4) motility should also be the same or increase. If the ejaculates do not meet these criteria, one or the other or both ejaculates are probably not
representative of what the stallion is capable of producing. If the discrepancy cannot be explained, the collections should be repeated on the same or the next day.

b. Gross Appearance:

Normal stallion semen is off-white, opaque and more watery than bull semen. Blood is deleterious to sperm and will give a pink to red color to the sample when present in a significant amount. If there is a question concerning its presence, any of the common laboratory tests for hemoglobin can be used. They are slightly positive without blood. Urination during or immediately after ejaculation results in semen which has an amber color and elevated pH.

Hemospermia can occur as a result of urethral erosions resulting from stallion rings or trauma, bacterial or viral urethritis, puncture wounds due to a breeding stitch in the mare, (86,87) seminal vesiculitis, (17,40) Habronemiasis of the urethral process, the migratory parasite - Strongylus edentatus (59) and warts.

c. Initial Motility - Total and Progressive:

The estimate of initial motility is made with undiluted, raw semen within five minutes of ejaculation. In general, the lower the percentage of progressively motile sperm, the lower the conception rate (CR) of that stallion will be, provided he is bred to a sufficiently large number of fertile mares. (45) semen in which there is consistently no motile sperm is expected to be sterile. It should be remembered that subjective motility estimates are just that. (See discussion under Sperm Morphology - B5c) Because the estimation of both total and
progressive motility is a subjective exercise we recommend that the estimate for percent of progressive motility be compared to the percent of sperm that have midpiece and tail abnormalities. When these 2 figures are substantially different, the percent with normal midpieces and tails be used as the percent of sperm that are progressively motile in determining the number of useful sperm in an ejaculate. The number of useful sperm being defined as the number of morphological normal, progressively motile sperm.

d. **pH:**

The normal pH range for stallion semen is 7.2 to 7.6. The second ejaculate tends to have a higher pH than the first. The pH should be estimated within ten minutes of ejaculation. Samples over 7.5 may be normal but such a pH should alert the examiner to seek an explanation. Two common potential sources of alkaline contamination are soap and urine. An elevated pH may also occur with inflammatory reactions. In bacterial infections of the excurrent duct system, the pH has been observed to go as high as 8.2.

e. **Sperm Morphology:**

The normal stallion spermatozoan has a head 5.8-6.5 μm long x 3 μm wide, thus being about 1/3 the size of the bull and boar. The head is about the diameter of an erythrocyte. The midpiece is 9-10 μm long by 0.6 μm in diameter and should not contain either proximal or distal cytoplasmic droplets. The tail is 40-70 μm long. In contrast to other domestic species, an abaxial attachment of the midpiece to the head is as normal as an axial one. In stained smears the head is asymmetrical with a less
concave surface on the side of attachment of the midpiece.\(^6\)

The cause of specific sperm abnormalities is not known. Therefore, it is not yet possible to use morphologic criteria as an aid in diagnosing specific diseases. Furthermore, it is not known whether morphologically abnormal sperm have an adverse affect on normal sperm. It is assumed, as in other species, morphological abnormalities can arise in the testes, in the epididymides or other parts of the excurrent duct system as well as in vitro.

A few specific morphological defects have been described which occur to such an extent that they lower fertility\(^{15}\) or lead to sterility.\(^{46}\)

The comparison of sperm morphology counts between ejaculates over time is often extremely useful in evaluating the progress of testicular degeneration or other disease.

For purposes of fertility evaluation, it is essential to determine the total number of morphologically normal sperm present in representative ejaculates and not be concerned with the percentage that are abnormal.

f. Sperm Numbers:

The number of sperm present in an ejaculate is determined by a wide variety of factors, including the size of the testicles, the season of the year, the frequency of use, the storage capacity of the extragonadal sperm reserves and the completeness of ejaculation. Add to this the number of agents, including drugs, that can decrease sperm numbers and it becomes clear that there will be a wide range in levels of innate fertility in the stallion population based on sperm numbers alone.
It is expected that the number of sperm per ejaculate will vary widely within and between stallions over time. Therefore, it is important to take all these factors into consideration when evaluating and prognosing stallion fertility.

Concentration of sperm by itself is of little value and is determined because it is essential for determining total sperm numbers. A critical factor in fertility is the total number of morphologically normal, progressively motile sperm ejaculated - regardless of concentration.

Sperm numbers can be accurately estimated after sperm motility has ceased so it is not necessary to determine concentration and volume promptly.

The concentration should be reported in #/cc, as from spectrophotometer, not #/mm³, as with hemocytometer. The conversion factor for converting mm³ to cc's is 1000.

g. Longevity of Motility in Raw Semen:

Sperm samples from fertile stallions generally maintain at least 10% progressive motility for six hours when stored under the conditions stated in Section A 8 c.i. This is not a rigid rule in regard to duration of motility but has been very useful in developing a presumptive diagnosis of short longevity of sperm in the female tract in stallions of low fertility and otherwise normal semen. Longevity of motility of two hours or less usually signifies a stallion which will have a subnormal conception rate under ordinary conditions when bred to a full book of mares, i.e., 40 or more with natural breeding or 120 or more if bred artificially. Six stallions with sperm of short longevity (≤2 hours)
have exhibited much improved conception and foaling rates when bred consistently within six hours of ovulation. Unfortunately this often means breeding at night.

h. **Longevity of Motility in Extender:**

The test is run in concert with estimation of longevity in raw semen. The sperm usually remain motile longer in extender. The reason for performing this test is to determine how much longer sperm remain active under these conditions when longevity is subnormal in raw semen. If there is a drastic difference, utilizing extender can be a therapeutic approach to management of stallions with fertility problems attributable to short longevity of sperm. It should be done with 2 or more extenders.

i. **Washing Sperm:**

Washing of sperm is not performed with every ejaculate but is performed on specimens of low motility so as to determine if the low motility is due to a defect in the sperm or if a seminal plasma effect exists. When motility is improved by washing, it is suspected that the motility of such sperm is suppressed by some factors in the seminal plasma and the observation provides grounds for a therapeutic approach to the problem if artificial breeding (AB) can be used, providing no genetic implications are involved. If AB can not be used, the extender can be placed in the uterus just prior to breeding.

j. **Agglutination (clumping):**

The cause of agglutination as well as its significance in regards to fertility are unknown. Currently its main effect is considered to be its interference with evaluation of motility. That effect is rarely
a problem providing estimations are made immediately after semen collection. The presence of blood or debris will often result in agglutination of sperm particles.

k. Bacteria:

The organisms commonly recovered from normal stallions on urethral swabbing and/or in semen are as follows:

1. a. B hemolytic streptococci
   b. Non-hemolytic streptococci
2. a. Hemolytic staphylococci
   b. Non-hemolytic staphylococci (Micrococci)
3. Coliforms
   a. E. coli
   b. Proteus sp.
   c. Enterobacter sp.
   d. Klebsiella
4. Pseudomonas sp.
5. Corynebacterium sp.
6. Various fungi and yeasts (Candida)

Most, if not all, of these are surface contaminants. The fungi are not known to infect the stallion genital system but several of the bacteria are known to do so. The following organisms, under the proper circumstances, can be venereally transmitted to mares: coliforms, B hemolytic streptococci, Pseudomonas aeruginosa, and Klebsiella pneumoniae. (19,20,21,30,35,37,38,42,43,58,76,84)

Hemophilus equisimilis, the organism of contagious equine
metritis, not only has limited geographical distribution but causes a specific venereal disease in which the stallion is a lesionless carrier. \(^{(72)}\)

To demonstrate that the excurrent duct system or one or more of the accessory genital organs of a stallion is infected can be difficult. \(^{(17)}\) To do so necessitates that the suspected, offending organism be found in essentially pure culture during repeated samplings.

The role of stallions in venereal transmission of viruses is poorly understood. The 2 viruses which have been shown to be a potential danger in this regard is the one causing equine infectious anemia and the one causing coital exanthema which is only a transient problem. During the acute, but not the inapparent, phase of EIA semen of an afflicted stallion contains infectious virus \(^{(16)}\) thereby being capable of transmitting the disease. Although the stallion in the acute phase with virus in the semen was not used for breeding, the disease has been produced in mares by breeding with semen to which virus has been added in levels found in spontaneous cases. \(^{(16)}\) Thus, evidence of infection (positive Coggins test; isolation of virus) constitutes grounds for rendering the stallion an UNSATISFACTORY PROSPECTIVE BREEDER.

Although the virus of equine viral arteritis causes scrotal edema, it apparently has no direct effect on the testicles and very little, if any, indirect thermal effects. \(^{(12)}\)

C. OVERALL STALLION EVALUATION

Fertility evaluation of stallions is not a precise science because of the numerous variables affecting the results, that is the pregnancy and foaling rates. The major variables affecting the results are the innate
fertility of the band of mares the stallion is bred to, the overall management of both the stallion and the mares, the veterinary management, and last, but not least, the quality of the performance of the tests as well as the quality of the interpretation of all findings.

Once the history has been taken and the various examinations, tests, and analyses have been made there may be difficulty in interpreting their importance in relation to the stallion's potential fertility. There is certainly no uniformity of opinion of what the optimal or minimal values of these various measures are as they relate to fertility, except in the general way that numbers of morphologically normal, progressively motile sperm have a quantitative relationship to fertility. Only one report specified the criteria for declaring a stallion to be a satisfactory, questionable or unsatisfactory prospective breeder.\(^{(49)}\) For this and other reasons the criteria set forth here should be considered satisfactory at this time but will be modified as new knowledge and additional experience is gained, particularly with the evaluation of stallions at Daily Sperm Output (DSO).

The committee feels that the critical factor regarding semen quality, assuming no defects in the seminal plasma, is the number of morphologically normal, progressively motile sperm the stallion is able to deliver into a natural or artificial vagina. The numbers of morphologically abnormal or non-motile forms is relatively unimportant concerning stallion fertility and are important only in calculating the number of normal sperm. Because of this it is not surprising to find no significant association between fertility and motility and morphological parameters among stallions\(^{(88)}\) when they are bred to a small number of mares and each receives a surplus
of morphologically normal, progressively motile sperm. When this occurs the results depend on other factors.

To consider a stallion to have sufficient fertility to have a book of 40 mares, or 120 if artificial breeding is used, the following guidelines are offered. It is worthwhile to emphasize that judgement must be used in applying these guidelines - taking into consideration all variables involved.

For less than a 40 or 120 book of mares the requirements, particularly in sperm numbers, will be proportionately less demanding.

For a full book the stallion should have the following qualities:

1. The stallion should demonstrate good libido, as indicated by short reaction time, to move freely, to find and mount the mare, to make introduction and to promptly ejaculate semen free of urine and/or blood.

2. The penis must be of normal size and shape and be free of lesions of an inflammatory nature.

3. The bacteria recovered from the semen and 2 urethral swabs should be inconsistent in type and reduced in number after ejaculation. There should be no colonies of the organism of Contagious Equine Metritis. In addition, multiple pure cultures or an unexplained increase in colony count on the second ejaculate are considered suggestive of reproductive tract infection and necessitate further investigation and clarification.

4. There should be no indication of equine infectious anemia as indicated by a negative Coggins test.

5. There should be 2 scrotal testicles and epididymides of palpably normal size, shape and texture.

6. The stallion should have the potential ability to ejaculate at least \(1 \times 10^9\) morphologically normal, progressively motile sperm in the second ejaculate each month of the year. (Sperm concentration \(\times\) semen volume \(\times\) \(\%\) morphologically normal sperm \(\times\) \(\%\) progressively motile sperm)

Beware of a total scrotal width of 8 cm or less. A stallion with such small testicles may fulfill the above criteria after a week of sexual
rest but would likely lack sufficient sperm producing capacity to qualify after reaching the level of DSO.

In attempting to predict whether the stallion can achieve the above level of production one must consider the effect of season since sperm production peaks in June and is at its lowest in December\(^{(67)}\) in the northern hemisphere.

The following table gives the minimal number of morphologically normal, progressively motile sperm which must be present in the second ejaculate for each month of the year in order for the horse to be able to produce 1 billion in December, the month of lowest production. The added provision is that both first and second ejaculates meet the requirements of representativeness. (See Section B.5,i.)

<table>
<thead>
<tr>
<th>Month</th>
<th># x 10(^9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jan.</td>
<td>1.2</td>
</tr>
<tr>
<td>Feb.</td>
<td>1.7</td>
</tr>
<tr>
<td>Mar.</td>
<td>1.8</td>
</tr>
<tr>
<td>Apr.</td>
<td>1.8</td>
</tr>
<tr>
<td>May</td>
<td>2.0</td>
</tr>
<tr>
<td>June</td>
<td>2.2</td>
</tr>
<tr>
<td>July</td>
<td>1.8</td>
</tr>
<tr>
<td>Aug.</td>
<td>1.7</td>
</tr>
<tr>
<td>Sep.</td>
<td>1.2</td>
</tr>
<tr>
<td>Oct.</td>
<td>1.2</td>
</tr>
<tr>
<td>Nov.</td>
<td>1.2</td>
</tr>
<tr>
<td>Dec.</td>
<td>1.0</td>
</tr>
</tbody>
</table>

If a stallion produces less than the above morphologically normal, progressively motile sperm, for any respective month, he should be considered a QUESTIONABLE OR UNSATISFACTORY PROSPECTIVE BREEDER, depending upon the severity of the problem.

If he does not meet these requirements the examination should be repeated every 60 days until he meets the criteria or else consider him less
than a satisfactory breeder. Some stallions will fail these tests for as long as twelve months yet finally pass.

If a horse meets these criteria he is classified as a SATISFACTORY PROSPECTIVE BREEDER FOR 40 (120) MARES. If he is borderline in 2 or more criteria he is considered a QUESTIONABLE PROSPECTIVE BREEDER for 40 (or 120) MARES. It is recommended that a re-examination be conducted at a later date, particularly if there is good chance of improvement. If the horse is very low in 2 or more criteria or has severe permanent shortcomings he is considered an UNSATISFACTORY PROSPECTIVE BREEDER for 40 (or 120) MARES.

It should be emphasized that the ultimate performance of the stallion will depend not only on the number and quality of sperm produced but also factors which cannot be measured in stallion evaluation, that is a multitude of management factors, veterinary care as well as the number and fertility of mares served.

These are considered to be conservative criteria which assure the owner or buyer of a stallion that there will be adequate quality of sperm to give each mare of the 40 or 120 book a reasonable chance to become pregnant provided management is adequate. There is provided a bit of a cushion in recognition of the fact that mares presented to the stallion, in breeds with a universal January 1 birthday (in the northern hemisphere: August 1 in the southern hemisphere) are not presented at a uniform rate but cluster in the months of May in the northern and November in the southern hemispheres; in addition, during this time there will be occasions when as many as 10% of a stallion's book can be ready to be bred on the same day. These criteria have been used in several laboratories and practices for several years and have
have proved to be adequate in assuring the ability to handle the afore-
mentioned minimal books.

The first edition of the Manual has been developed to:

1. Provide a guide to and set of procedures for estimating the
relative prospective fertility of stallions. The procedures selected can
be properly conducted by any veterinarian without elaborate equipment.

2. Provide description of how these procedures and tests are to be
conducted.

3. Provide criteria for interpreting the results of the procedures.

The need has long existed for published guidelines on stallion fer-
tility evaluation that can be used worldwide. It is anticipated that these
guidelines will be periodically revised.

The guidelines presented have been developed for veterinarians who
are called upon to evaluate stallions which are expected to have a full book
of mares (ie 40 for natural service or 120 for artificial breeding). In ad-
dition to criteria given here, when available, advantage should be taken of
previous reproductive performance in establishing the size of the book of
stallions providing management conditions are the same. The Committee also
realizes that the buyers of some stallions intend to book double, and more,
the number of mares noted above. Whether a particular stallion can success-
fully be bred to such large numbers of mares will depend not only on his
intrinsic seminal, physical and behavioral capacities but importantly on how
the stallion and his mares are managed. The veterinarian must be extremely
cautious in certifying that a stallion can successfully handle such large
books.
It is also realized that some stallion owners will wish to book a horse to fewer than 40/120 mares. If the stallion in question does not have the qualifications to meet the standards for 40/120 mares, he can still be classified as a SATISFACTORY PROSPECTIVE BREEDER FOR UP TO ___ mares. That is, a specifically designated number of mares which should be determined by his physical, behavioral and seminal qualifications. In other words a Specifically Qualified Satisfactory Prospective Breeder,
ACKNOWLEDGEMENTS

In the interest of developing a manual that would have international acceptance the Committee sent one or more drafts to colleagues overseas as well as in the United States.

The Committee appreciates and acknowledges the comments offered by the following persons. Recognition of many of their comments has been made by incorporation into the manual. Those not included have not been overlooked. The Committee takes full responsibility for what is included.


One or more drafts were sent to 9 others who failed to respond to the request for comments.

We are deeply indebted to the typists, Mrs. P. Brewer, Mrs. N. Wynne and Mrs. M. Armstrong, who worked under trying conditions, to the artist, Ms. Erlene Mitchner and to the photographer, Mr. Charles Tucker.
E. BIBLIOGRAPHY


BIBLIOGRAPHY (continued)


F. APPENDICES

1. Stallion Fertility Form

2. a. Work Sheet for Sperm Longevity of Motility
   b. Work Sheet for Semen Morphology

3. Equipment and Supplies

4. a. Diagram of Penis  c. Diagram of Prepuce
   b. Diagram of Head of Penis

5. Buffered Formal Saline Formula

6. Eosin Nigrosin Stain

7. India Ink

8. Giemsa Stain

9. Wright's Stain

10. Hemocytometer Counting of Sperm
     b. Using Serological Pipettes

11. Spectrophotometric Counting of Sperm

12. Spermatozoon Morphology
   a. Legend  b. Diagram

13. Diagram of Testicle and Epididymis

14. Diagram of Internal Genital Organs

15. Diagram of Pelvic Urethra

16. a. Technique of Washing Sperm
     b. Extenders for Estimating Longevity of Motility

17. Estimation of Motility and Longevity of Motility

18. CEM Code of Practice for Stallion
STALLION FERTILITY EVALUATION

<table>
<thead>
<tr>
<th>TESTS</th>
<th>1ST EJACULATE</th>
<th>2ND EJACULATE</th>
<th>3RD EJACULATE</th>
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<tbody>
<tr>
<td>Manner, Mounts, Time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume, Total (Cry Free)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gross Appearance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Motility (Total/Progressive)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH (Method)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration (x 10^6/ml) (Method)</td>
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<td></td>
</tr>
<tr>
<td>Total Number of Sperm (x 10^9)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total # Sperm x % Prog Motile x % Morph Normal (x 10^9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Normal Sperm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Abnormal Acrosomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Abnormal Heads</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>% Detached Heads</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Proximal Droplets</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>% Distal Droplets</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>% Bent or Coiled Midpieces</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Other Midpiece Abnormalities</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>% Hairpin or Bent Tail</td>
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</tr>
<tr>
<td>% Coiled Tails</td>
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<td></td>
<td></td>
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<tr>
<td>Other Cells</td>
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<tr>
<td>VIABILITY (hrs to 10% prog motility):</td>
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<td></td>
</tr>
<tr>
<td>Raw at _____ °C</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Extender( ) at _____ °C</td>
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<td></td>
</tr>
<tr>
<td>Extender( ) at _____ °C</td>
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<tr>
<td>CULTURE AND SENSITIVITY:</td>
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<tr>
<td>Pre-ejaculate urethra</td>
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</tr>
<tr>
<td>Post-ejaculate urethra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Semen</td>
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<td></td>
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</table>

(this form has two sides)
GENITAL PHYSICAL FINDINGS:

<table>
<thead>
<tr>
<th>(measurement technique = )</th>
<th>LEFT</th>
<th>RIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>testicle - length x width x height (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>palpation of testicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>epididymis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>internal ring (size)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>seminal vesicles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ampullae</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total Scrotal Width | Prostate | Penis

BEHAVIORAL FINDINGS:

<table>
<thead>
<tr>
<th>LIBIDO</th>
<th>ERECTION (time)</th>
<th>MOUNTING (time)</th>
<th>INTROMISSION</th>
<th>EJACULATION</th>
</tr>
</thead>
</table>

OTHER TESTS PERFORMED (Include Date and Results):

1. E.I.A. date____________ results: positive__ negative__
2. 
3. 

GENERAL PHYSICAL FINDINGS & IDENTIFYING MARKS: PHOTO

HISTORY:

REMARKS:

Veterinarian________________________
Telephone___________________________
Address______________________________

:sma6883
ANIMAL ID ____________

SPERM LONGEVITY OF MOTILITY WORKSHEET

<table>
<thead>
<tr>
<th></th>
<th>1ST EJACULATE</th>
<th></th>
<th>2ND EJACULATE</th>
<th></th>
<th>3RD EJACULATE</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DATE AND HOUR</td>
<td></td>
<td>DATE AND HOUR</td>
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<td>DATE AND HOUR</td>
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<td>DATE AND HOUR</td>
</tr>
<tr>
<td>ELAPSED TIME</td>
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<td>ELAPSED TIME</td>
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<td>ELAPSED TIME</td>
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<td>4°C 22°C</td>
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<td>4°C 22°C</td>
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<td>4°C 22°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EXTENDER</td>
<td></td>
<td>EXTENDER</td>
<td></td>
<td>EXTENDER</td>
<td></td>
</tr>
</tbody>
</table>
### APPENDIX 2B
### SEMEN MORPHOLOGY WORKSHEET:

#### I. NORMAL

#### II. ABNORMALITIES

##### A. HEAD
1. Abnormal acrosome
2. Pyriform
3. Round
4. Elongated, narrow
5. Microcephalic
6. Macrocephalic
7. Double
8. Atypical stainability
9. Hourglass
10. Scalloped margins
11. Detached normal heads

   **SUB-TOTAL**

##### B. MIDPIECE
1. Abaxial
2. Proximal droplets
3. Distal droplets
4. Atypical location of drop
5. Double
6. Thickened
7. Kinked
8. Coiled
9. Broken midpiece
10. Damaged
11. Single loop
12. Double loop
13. Filamentous

   **SUB-TOTAL**

##### C. TAIL
1. Coiled
2. Single loop
3. Double loop
4. Bent tail
5. Broken tail
6. Filamentous

   **SUB-TOTAL**

##### D. OTHER CELLS
1. Medusa
2. Epithelial
3. Leukocytes
4. Erythrocytes
5. Spermatocytes & spermatids

   **SUB-TOTAL**
APPENDIX 3
EQUIPMENT AND SUPPLIES FOR
STALLION FERTILITY EVALUATION

a. Nishikawa Artificial Vagina

Tom Scott, Medical Products
457 Ginger Avenue
Haywood, CA  94541

No phone
Price:  $350.00

b. Missouri Artificial Vagina

Nasco
901 Janesville Avenue
Fort Atkinson, WI  53538
Telephone:  800/558-9595
Model:  C 6248 N
Price:  $99.50

Rubber Liner (C6250N) $80.50

Edwards Agri-Supply
POB 65, 201 Lynn Ave.
Baraboo, WI  53913
Telephone:  800/356-5813
Price:  $281.80
Liner:  $228.85

C. Colorado Artificial Vagina

Lane Mfg. Co., Inc.
5560 E. Pacific Place
Denver, CO  80222
Telephone:  303/758-5370
Price:  $512.25

Edwards Agri-Supply
see b.
Price:  $451.50

D. Sterile, water soluble lubricant without disinfectant

Example:  KY
Mfg. Johnson & Johnson
New Brunswick, NJ
Source:  Most Medical Supply Houses
APPENDIX 3 (continued)

e. Whirl Pak Bags

NASCO

see b.

Missouri AV Bag
B 679 (6 oz)  
Price: $46.50/1,000

Nishikawa AV Bag
B 736 (18 oz)  
Price: $65.90/1,000

f. Evenflo Disposable Bottles

Evenflo Products Co.
Ravena, Ohio 44266

g. Playtex Disposable Bottles

International Playtex, Inc.
PO Box 1400
Dover, Delaware 19901

h. A.V. Adapter

Summit Hill Laboratories
Avalon, NJ 08202

Distributed by Butler Co.
400 Babylon Rd.
Horsham, PA

Phone: 215/674-3555

Price: $20.00

Distributed by Butler Co.
Columbus, OH 43228

Phone: 800/848-5983

i. Teflon Fitting

Mr. Jim Harvey
Lana Lobell Farm
Hanover, PA

j. Tapered Breeding Roll & Neck Pad

Pinkston's Turf Goods, Inc.
125 N. Broadway
Lexington, KY

Phone: 606/252-1560

Roll Price: $65.00

Neck Pad Price: $225.00

$300.00 X-long
k. Condoms (Breeding Bags)

Jorgensen Labs
2198 Wiser St.
Loveland, CO 80539
Price: $7.20

Dean Rubber Co.
1601 Iron City, Box 12344
N. Kansas City, MO 64116
Price: $3.00

1. In-Line Milk Filter

Agway Co.
Syracuse, NY
and other dairy supply houses

To Prepare:
Cut 12 inch liners into 3 pieces that are 4 inches long. Remove glued end. Machine stitch 1 end. Sterilize.

m. Sterile Plastic Beaker - 50 ml with lid

Falcon Plastics #4015 - most laboratory supply houses
Price: $20.85/100

n. Pasteur Pipettes

Fisher Scientific
Cat. #13-678-20A (Kimble #72000)
Price $14.69/720

A.H. Thomas
Cat. # 7760-A10
Price: $10.00/360

o. Wooden Applicator Sticks

Fisher Scientific
Cat. # 01-340
Price: $12.00/box of 864

A.H. Thomas
Cat. # 2942 C 10
Price: $8.32/864

p. Incubator

Fisher Isotemp Series 100 Gravity Convection (smallest model) 1.3 cu. ft.
Catalog #: 115V 11-683-116D
Price: $357.00

A.H. Thomas
Cat. #: 6118-L-25
Price: $379.00

q. Slide Warming Tray - 6762-510

Arthur H. Thomas Co.
Vine St. @ Third, PO Box 779
Philadelphia, PA 19105
Price: $175.00

Fisher Scientific
Model: 12-594
Price: $260.00
r. pH Meter - Accumet 800 pH/mv meter

Fisher Scientific
Catalog #: 13-636-800
Price: $495.00

A.H. Thomas
Corning Model 7
Cat. #: 4135 C 10
Price: $685.00

s. Short Range pH Paper

Fisher Scientific
pH Indicator Paper A-983
Price: $5.25 for 3

A.H. Thomas
Cat. #: 3109 B 30
Price: $4.10

t. Hemocytometer

Fisher Scientific
Cat. #: 02-671-5
Price: $45.00

A.H. Thomas
Cat. #: 2936-M 10
Price: $42.00

u. Spectronic 20

Fisher Scientific
Model No. 07-143-1
Price: $875.00

A.H. Thomas
Model No. 8420-H65
Price: $875.00

v. 6 ml Sterile Tube for Longevity and BFS

BBL-Falcon
Catalog #: 2054
Price: $30.54/500

Most laboratory supply houses

w. IEC Clinical Centrifuge

Arthur H. Thomas
Cat. No. 2509-A05
Price: $350 for drive unit (heads, shields, rings extra)

x. Scrotal Caliper

Lane Mfg. Co.
5560 E. Pacific Place
Denver, CO 80222
Telephone: 303/758-5370
Cat. No. 33-03
Price: $52.00
y. Fiberoptic Scope

1. Olympus Corp. of America
   4 Nevada Drive
   New Hyde Park, NY 11042
   Telephone: 516/488-3880
   Model: GIF-P-(1000 mm working length)
   Difficult to obtain

2. Olympus
   Model: GIF-XP (1135 mm working length)
   (7.8 mm diameter)
   Price: $7400.00

z. Falcon 50 ml, conical, graduated testtubes with positive seal cap

   Falcon Plastics
   Catalog #: 2070
   Price: $159.35/case of 500

Falcon, Fisher and Thomas have national distribution.

Home offices and phone numbers are as follows:

Oxnard, CA  93030  711 Forbes Avenue  Pittsburgh, PA 15219  Vine Street at Third
               412/784-2600  PO Box 779  Philadelphia, PA 19105
               215/574-4555
APPENDIX 4a

PENIS LEGEND

a - OUTER LAMINA OF PREPUTIAL FOLD;  b - PREPUTIAL RING;  c - INNER LAMINA OF PREPUTIAL FOLD;
d - ATTACHMENT OF INNER LAMINA OF PREPUTIAL FOLD TO PENIS;  e - CORONA GLANDIS;  f - CLANS PENIS;  
g - URETHRAL PROCESS;  h - COLLUM GLANDIS;  i - FREE PART OF PENIS;  j - INTERNAL LAMINA OF
EXTERNAL FOLD OF PREPUCE;  k - SCROTUM
LEGEND FOR SAGITAL SECTION OF END OF PENIS

a. GLANS PENIS; b. CORPUS CAVERNOSUM; c. URETHRAL SINUS (DIVERTICULUM); d. FOSSA GLANDIS;
e. URETHRAL PROCESS; f. CORPUS SPONGIOSUM
LEGEND FOR SAGITAL SECTION OF PREPUCE

a. PREPUCE PROPER (INTERNAL PORTION OF PREPUCE); b. PREPUTIAL CAVITY; c. PREPUTIAL RING; d. GLANS PENIS; e. PREPUTIAL ORIFICE; f. URETHRAL PROCESS; g. SHEATH OR EXTERNAL FOLD OF PREPUCE; h. INNER LAMINA OF PREPUCE PROPER; i. OUTER LAMINA OF PREPUCE PROPER; j. INNER LAMINA OF SHEATH (EXTERNAL FOLD OF PREPUCE); k. OUTER LAMINA OF SHEATH
APPENDIX 5

BUFFERED FORMOL SALINE (BFS)

\[
\begin{array}{ll}
\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O} & 6.19 \text{ gm (4.93 GmNa}_2\text{HPO}_4) \\
\text{KH}_2\text{PO}_4 & 2.54 \text{ gm} \\
38\% \text{ formaldehyde} & 125.00 \text{ ml} \\
\text{NaCl} & 5.41 \text{ gm} \\
\text{Aq. dist. qsad} & 1000.00 \text{ ml}
\end{array}
\]

Add five drops of well mixed semen to the surface of 2 ml of BFS.
Mix by rotation of vial or tube.
Place a drop or two of well mixed diluted semen under coverslip and examine under oil immersion using phase contrast or phase interference microscopy.
Practitioners lacking these types of microscopes can get a theriogenologist at one of the universities to perform this examination by shipping preserved sperm.

An eosin nigrosin stain is available through the Society for Theriogenology. It has proven satisfactory for morphological evaluation of spermatozoa.

Smears are prepared on warm slides (35-37°C) by placing a small drop of stain on the slide. A small drop of semen is then added to the stain and mixed. A second slide is used to make the smear as one would prepare a blood smear. The smear should be quickly dried. Cells are counted under oil immersion.

This stain has been touted as a live-dead stain with dead sperm taking up the eosin. This aspect of the stain is capricious in that humidity drastically effects the percentage of sperm that take the sperm.

It is otherwise an excellent background stain.

Available from the Society for Theriogenology, Association Building, Ninth and Minnesota, Hastings, Nebraska 68901.
APPENDIX 7

INDIA INK (AN OUTLINE STAIN)

Use fine-grained India ink. Coarse-grained inks are inadequate.*

1. Using a Pasteur pipette or preferably an applicator stick, place a small drop of ink on the end of a slide. The proper size will spread to between two and three millimeters.

2. Add a small drop of semen to the ink. This can be done by dipping an applicator stick in semen, then placing it in the ink on the slide and then mixing the two.

   The mixture is then spread with a second slide as with blood smears.

   Prompt microscopic examination will reveal if sperm concentration is too high or low for efficient evaluation. Avoid overlapping sperm.

3. The advantage of this stain is that it is quick and easy. It also will reveal acrosomal defects whereas positive stains usually fail.

4. Air dry and examine under oil immersion.

* "Pelican" is a brand of fine-grained India ink.
APPENDIX B

GIEMSA STAIN

SOLUTIONS

Jenner Solution (Stock - Harleco #642 or other source)*
   or
Jenner stain (Harleco or other source)  1.0 gm
   Alcohol, methyl  400.0 ml

Jenner Solution (Working)

Jenner solution (stock)  25.0 ml
Distilled water  25.0 ml

Giemsa Solution (Stock) (Harleco #642 or other source)*

Giemsa Solution (Working)

Giemsa solution (stock)  2.5 ml
Distilled water  50.0 ml
Make fresh, do not re-use.

1% Glacial Acetic Water Solution

Glacial acetic acid  1 ml
Distilled water  100 ml

STAINING PROCEDURE

1. Air dry smear.

2. Methyl alcohol, two changes for 3 minutes each.

3. Working Jenner solution for 6 minutes.

4. Working Giemsa solution for 45 minutes.

5. Handle each slide individually in this and subsequent steps. Differentiate in glacial acetic water solution (3 quick dips) then check microscopically for well differentiated nuclei.

6. Rinse in distilled water.

7. Dehydrate quickly in 95% alcohol, absolute alcohol, and clear with xylene, two changes each.

8. Mount with Permount or Histoclad.
RESULTS

APPENDIX 8 (continued)

Nuclei - blue
Cytoplasm - pink to rose
Bacteria - blue

REFERENCE

Manual of Histologic Staining Methods
McGraw-Hill Book Co.

* Harleco
480 Democrat Road
Gibbstown, NJ 08027
APPENDIX 9

WRIGHT'S STAIN

SOLUTIONS

Wright's Stain*

Buffer Solution*

Sodium phosphate, dibasic 0.3 gm
Sodium phosphate, monobasic 0.7 gm
Distilled water 100 ml

METHOD

1. Make thin smear of semen - as for blood smear. Do not let sperm overlap.

2. Lay face up and horizontally until dry.

3. Cover smear with Wright's staining solution (about 15 drops).

4. Let sit for 1 minute and then add twice the volume of distilled water, or instead of water, a 1:6 dilution of the above buffer stock solution. You should see a metallic sheen on the solution before proceeding with step 5. Allow the water or buffer and dye mixture to remain on the slide for at least 2 minutes.

5. Drain off and rinse with diluted buffer or distilled water.

6. Blot dry, and mount in synthetic mounting medium.

EXAMINE UNDER OIL IMMERSION

* Harleco
480 Democrat Road
Gibbstown, NJ 08027
APPENDIX 10

A. STANDARD HEMOCYTOMETER SPERM COUNT

The hemocytometer sperm count is made in a Spencer Bright-Line hemocytometer (or Neubauer counting chamber).

1. Mix the sample carefully and thoroughly before sampling.
2. Use a Thoma RBC pipette.
3. Ejaculate is then drawn into a red cell pipette to the 1 ruling, being careful not to go beyond this point (the sperm will adhere to the barrel of the pipette).
4. Draw normal, isotonic saline (0.85) or water to the 101 mark. Be careful to not go beyond this point as it will greatly amplify error. This will form a 1:100 dilution.
5. Holding pipette between thumb and forefinger, gently shake up and down, not from end to end, for approximately one and one-half minutes. Shaking pipette from end to end has a tendency to force liquid out of diluting chamber. Also, an automatic pipette shaker may be used, but severe vibration may cause undue fragmentation of sperm.
6. Three to five drops are then expressed from the tip of the pipette and discarded. Then both chambers of the hemocytometer are filled as in counting blood cells. It is very important not to flood the chamber causing the sample to overflow into the moat. Place the chamber in a covered petri dish with moistened cotton or filter paper for ten minutes to allow spermatozoa to settle.
7. Count only heads in five small squares in the large, central square (red cell area) of Neubauer chamber (see figure on following page).
APPENDIX 10 (continued)

7. (continued) Sperm can be counted in five squares of the twenty-five total, either diagonally, vertically or horizontally (using that which gives the best distribution of cells).

8. To determine the number of sperm per ml of ejaculate, multiply the number of sperm counted by five million ($5 \times 10^6$).

9. For samples with very low concentration, use the Thoma WBC pipette and the white blood cell counting procedure. Draw semen to 0.5 mark and fill the chamber as above. Count the four large corner squares. In this case, to determine the number of sperm per ml, multiply the number counted by $50 \times 10^3$. In even less concentrated samples, draw semen to 1.0 mark. In this case, multiply the number counted by $25 \times 10^3$. 
APPENDIX 10 (continued)

B. HEMOCYTOMETER COUNT USING SEROLOGICAL PIPETTES (for 1:100 dilution)

1. Place 9 ml of formol saline into a clean test tube and add 1 ml of well mixed semen. Gently mix. This will yield a 1:10 dilution.
2. Place 9 ml of formol saline in second test tube and now add 1 ml of well mixed 1:10 diluted sample. This will yield a 1:100 dilution.
3. Mix sample extremely well without shaking, then using a pasteur pipette, fill counting chamber and count cells as previously discuss under A. - steps 6, 7, and 8.

C. HEMOCYTOMETER COUNT USING UNOPETTE* FOR PLATELET/WBC (1:100 dilution)

1. Using the pointed of the capillary guard, puncture the neck opening of the reservoir and remove tape.
2. Remove capillary pipette from guard and touch the pipette to the surface of well mixed semen. Capillary action will draw semen to capillary bore in neck of pipette.
3. Wipe excess sample from outside of capillary pipette being careful not to draw sample out of tube.
4. Squeeze reservoir to displace small amount of air. Insert pipette tip into neck of reservoir and into the diluent. Release pressure. Sample will be drawn into diluent.
5. Flush pipette tip by gently squeezing diluent in and out of pipette. Be careful not to expel any sample through top of pipette.
6. Mix by gently rocking reservoir.
7. Remove pipette and place on neck of reservoir in reverse position.
8. Discard first three to four drops through pipette by gently squeezing
8. (continued) reservoir.
9. Fill chamber wells and count cells as previously discussed under A. - steps 6, 7, and 8.

* #5855 Unopette for Platelet/WBC determination for manual methods is recommended for counting of horse semen. Ammonium oxalate kills spermatozoa yet does not cause agglutination.

B-D Becton-Dickinson, Rutherford, NJ
APPENDIX 11

OPERATING PROCEDURE FOR DETERMINATION OF STALLION SPERM CONCENTRATION WITH THE SPECTRONIC 20

1. Turn on Spec 20 and allow at least thirty minutes warm up time.

2. Ensure that you have adequate Spectronic 20 tubes. (One for blank plus two for each sample.)

3. Spec 20 tubes must be meticulously clean and free of scratches.

4. Add 7.6 ml of formol saline to two sample tubes.

5. Add 8.0 ml of formol saline to blank tube.

6. Add 0.4 ml of semen to sample tubes (dilution = 1:20) (0.4 cc in 8.0 cc).

7. Do not add semen to blank.

8. Adjust wave length control to 550 nanometers.

9. Adjust zero control so meter reads "0" (zero).

10. Wipe blank carefully with Kim Wipe.

11. Insert blank (Spec 20 tube with 8.0 ml formol saline) firmly into holder and close top.

12. Adjust light control so meter reads 100.

13. Remove blank and check 0 (readjust if necessary).

14. Repeat Steps 10 through 13 until you consistently get 0 and 100 respectively.

15. Mix sample thoroughly by gently rocking tube. Wipe off sample tube with Kim Wipe.


17. Close lid and record meter reading from %T scale.

18. Repeat Steps 15 through 17 for duplicate sample.

19. If readings from unknowns are greater than 1% transmittance, repeat dilutions.
20. If readings are within 1%, average %T.

21. If readings are between 20% and 80% T, go to calibration data and read appropriate concentration from standard curve.*

22. If reading is less than 20% transmittance, the semen is very concentrated and must be diluted further. Add 7.8 cc of formol saline to two more Spec-20 tubes and add 0.2 cc of semen. (dilution = 1:40) (0.2 cc in 8 cc). Reread samples and use 1:40 scale from standard curve and record sperm concentration.

23. If reading is more than 80% transmittance, the semen is very dilute.

24. Add 7.2 cc of formol saline to each of two sample tubes plus 0.8 cc of semen (dilution = 1:10) (0.8 cc in 8 cc).

25. Read and record value from 1:10 scale from standard curve.

26. If reading is still more than 80%, it must be counted with a hemocytometer. (Concentration is less than 21.95 x 10⁶ spermatozoa per cc.)

* The standard curve can be constructed using comparison between duplicate hemocytometer counts and Spec 20 %T readings. Curve is constructed using the following linear regression equation:

\[ \text{Optical Density} = 2 - \log \%T \]

Plot OD against hemocytometer count.
APPENDIX 12

TECHNIQUE OF MORPHOLOGICAL EVALUATION OF SEMEN

Most of the cells in semen will be sperm. In addition, there may be spermatocytes, spermatids, sloughed epithelial cells and leukocytes.

The purpose of the morphological evaluation of sperm is to determine what percentage are normal, to determine the types of abnormalities for their potential diagnostic value, and to evaluate the types and numbers of non-sperm cells for their contribution to the establishment of a diagnosis of problems or potential problems.

As noted in the text discussion, the percent of midpiece and tail abnormalities can be used to calculate the percent of sperm exhibiting progressive motility.

The spermatozoa are evaluated for their morphological normalcy. For this purpose a minimum of 200 properly preserved and processed spermatozoa are examined (Appendices 5, 6, 7). A form is provided for recording the abnormalities seen (Appendix 2b) along with a diagram of the common abnormalities for identification purposes (Appendix 12a, 12b).

It is important to remember that the objective is primarily to determine the percentage of normal sperm present and secondarily the percentage and types of abnormalities. Therefore, if a sperm has more than one abnormality, only one abnormality should be attributed to it. Otherwise there will be more abnormalities than sperm, which defeats the primary purpose intended here.

Non-sperm cells are best identified on air dried smears stained with either Wright's or Giemsa stain.

When there is a high number of abnormal sperm or there are questions about a stallion's fertility, it is advisable to evaluate 400 spermatozoa as well as any non-sperm cells present. This may require the help of specialists.
APPENDIX 12a

LEGEND FOR SPERMATOZOA

A. Normal
B. Double headed
C. Microcephalic heads
D. Pyriform heads
E. Scalloped heads
F. Detached heads
G. Droplets, distal, proximal and 2 unusual ones
H. Abnormal midpieces
I. Double tail
J. Single loop tails
K. Double loop tails
L. Damaged sperm
M. Ciliated epithelial cell
N. Sperm heads still in spermatid cytoplasmic membrane
O. Sperm heads out of, but attached, to plasmic membrane
P. Acrosomal defects
APPENDIX 13

TESTIS AND EPIDIDYMIS LEGEND

A. SPERMATIC CORD WITH VESSELS
B. DUCTUS DEFERENS
C. LIGAMENT OF EPIDIDYMIS (GUBERNACULUM)
D. BODY EPIDIDYMIS
E. HEAD EPIDIDYMIS
F. TAIL EPIDIDYMIS
G. APPENDIX TESTIS
H. PROPER LIGAMENT OF TESTIS
I. ENTRANCE TO EPIDIDYMAL BURSA
LEGEND FOR INTERNAL GENITAL ORGANS OF STALLION

A. BLADDER; B. ROUND AND LATERAL LIGAMENT OF BLADDER; C. GENITAL FOLD;
D. CAUDAL EXTENT OF PERITONEUM; E. SEMINAL VESICLE; F. UTERUS MASCULINUS;
G. AMPULLA; H. PROSTATE; I. PELVIC URETHRA; J. BULBOURETHRAL GLAND -
RIGHT WITH CONNECTIVE TISSUE COVERING, LEFT WITH COVERING REMOVED.
LEGEND FOR PELVIC URETHRA AND BLADDER

A - URETER; B - BLADDER; C - AMPULLA; D - SEMINAL VESICLE;
E - LOBE OF PROSTATE; F - SEMINAL COLICULUS; G - PROSTATIC DUCTS;
H - BULBOURETHRAL DUCTS; I - LATERAL URETHRAL GLAND DUCTS; J - DUCTS
OF AMPULLAE; K - DUCTS OF SEMINAL VESICLES
APPENDIX 16a
TECHNIQUE OF WASHING SPERM

The semen is collected from a properly washed and rinsed penis in a properly prepared artificial vagina as described in section using a Whirl-Pak bag (Appendix 3e) or other sterile container. Before inserting the penis into the AV it is diverted away from the mare or dummy so as many of the pre-ejaculatory secretions as possible are voided outside the AV. This maneuver not only prevents contamination of the penis by contact with the mare or dummy but the flushing by secretions minimizes the bacterial contaminants from the urethra.

The semen is poured through a sterile in-line milk filter (Appendix 31) into a sterile plastic beaker (Appendix 3m) in which the final volume of the gel-free semen is noted and recorded. The semen is evaluated according to Section

An aliquot of well mixed semen is then placed in a sterile, chemically clean test tube (Appendix 3 v or z) and an equal amount of extender added and mixed. The tubes are capped and centrifuged at 300 G's for 3 minutes.

(1500 RPM with 7.5 cm arm)

The supernatant is carefully poured off and the soft pellet is resuspended in an amount of extender equal to the original volume of the sample.

The tube is recapped with as little air space as possible and placed in a draft-free, dark location at room temperature.

Every 15' for the first hour, the sample is gently mixed without shaking, drop removed, placed on a prewarmed slide and covered with a prewarmed coverslip. The percentage of sperm undergoing progressive motility is estimated and
APPENDIX 16a (continued)

recorded. This process is continued until progressive motility declines to 10%. The intervals between samplings can be extended to one half or even one hour once it is evident that longevity of motility will be prolonged.
EXTENDERS FOR ESTIMATING LONGEVITY OF MOTILITY

I. Instant Non-fat Dry Milk

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4.9 gm</td>
</tr>
<tr>
<td>Penicillin, crystalline*</td>
<td>150,000 units</td>
</tr>
<tr>
<td>Dihydrostreptomycin, crystalline</td>
<td>150,000 micrograms</td>
</tr>
<tr>
<td>Sterile Distilled Water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

II. Instant Non-fat Dry Milk

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4.9 gm</td>
</tr>
<tr>
<td>NaHCO₃ (7.5%) (Abbott)</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Gentamicin sulfate (50mg/cc)</td>
<td>100 mg</td>
</tr>
<tr>
<td>Sterile Distilled Water</td>
<td>92 ml</td>
</tr>
</tbody>
</table>

(Be sure to add NaHCO₃ to milk mixture or to gentamicin before adding gentamicin. The latter is acidic and can coagulate milk protein.)

III. Skim milk/Polymyxin

a. Heat 100 ml non-fortified skim milk in double boiler for 10 minutes at 92°-95°C (198°-203°F)
b. Add polymyxin B sulfate, 1000 IU/ml
c. Freeze in 10 ml aliquots until used
d. Thaw to 19°C (85°F) before adding semen.

See reference 51
* Gibco Laboratories, 3175 Staley Road, Grand Island, NY 14072 800/828-6686
Penicillin/Streptomycin-Cat. No. 600-5145-Add 20 ml sterile saline to vial, mix, remove 15 ml of mixture and add to other constituents.

APPENDIX 17

ESTIMATION OF MOTILITY

Estimation of the percentage of sperm that is motile should always be done under ideal conditions for the sperm. In estimating initial motility, the sample should be removed from a well-mixed ejaculate with a clean, warm Pasteur pipette, placed on a pre-warmed (38°C) slide and covered with a cover slip at the same temperature. The estimates should be made within a few minutes of collection and all supplies should be ready before the collection is made.

The sample is then examined at low dry magnification (10x objective) and the total percentage of sperm that is motile is estimated and then the percentage of progressively motile sperm is estimated. Another useful method is to look at a field with the high dry objective (40x) and examine ten sperm while noting how many of the ten are motile and how many progressively motile. It takes training to look at the first ten one views because the eye tends to follow the more motile ones and tends to "avoid" the immotile ones. As soon as the ten are evaluated, switch to 10x and estimate total and progressive (T/P) motility and compare to the estimate made at 40x. Follow this procedure in many fields, at different locations of the sample. Repeat with numerous samples. With sufficient practice one will be able to make accurate estimates. The percentages derived with a 40x objective serve to "teach" one to make accurate estimates with the 10x. The advantage of examination with the 10x objective is that much larger fields will be examined. To assess motility accurately requires practice and experience. It would help the inexperienced clinician to spend time and learn from persons who do it routinely.

It is best not to dwell over one sample, but rather to make the estimates with dispatch and then resample. The light from the microscope is damaging to sperm so there will be a relatively rapid demise while on the microscope.
I. BACTERIOLOGICAL MONITORING METHOD

Culture swabs are to be placed in transport media immediately and taken to the laboratory as quickly as possible.

Stallions in which infection is known or suspected will have bacterial cultures taken from the sheath, the urethra, urethra fossa and pre-ejaculatory fluid if this can be obtained by the attending veterinarian before treatment is started. The cultures should be taken on separate swabs from the various areas.

II. TREATMENT OF STALLIONS

All treatments and examinations of mares and stallions will be performed or supervised by a licensed, accredited veterinarian. Any handling of the genitalia of Thoroughbred mares or stallions will be done with disposable gloves, wraps, and instruments properly sterilized by autoclave or comparable method. All pails will have disposable liners. Proper disposition of all gloves, drapes, liners, and instruments shall be accomplished directly under veterinary supervision.

This treatment must be carried out with the stallion in full erection and with the operator wearing disposable gloves and using disposable equipment.

A. Mechanically clean the external genitalia with clean, warm water.

B. Apply a chlorhexidine-containing surgical scrub (Nolvasan Surgical Scrub, Fort Dodge Laboratories, is suggested) liberally and, using sufficient water to obtain sudsing, cleanse thoroughly paying particular attention to the urethral fossa/sinus and the folds of the sheath.

C. Wash with clean warm water and dry.

D. Apply Furacin ointment liberally, insuring filling of the urethral fossa/sinus and penetration of the folds of the sheath.

E. Repeat the treatment daily for five days (series of five treatments).

F. Two test mares must be bred. To qualify as test mares, they must have two sets of no growth cultures from the cervix, clitoral sinus and clitoral fossa.
F. (continued)

At the time the qualifying swabs are taken, blood must be taken and subjected to complement fixation test.

The mares are test bred by the stallion seven or more days after his last treatment. The mares have additional sets of swab cultures made on post breeding days two, four and seven. A fourth set is taken on the post breeding estrus or if mare is pregnant she is aborted within twenty-eight days and a fourth set of swab cultures of the endometrium, clitoral fossa and sinus is made during estrus.

A second CF test is made between fifteen and forty days after breeding.