<u>Avoiding the Pitfalls and Making the Most of Diagnostic tests for Strangles</u>

Andy E Durham *BSc.BVSc.CertEP.DEIM.DipECEIM. MRCVS., RCVS and European Specialist in Equine Internal Medicine*

Liphook Equine Hospital, Portsmouth Road, Liphook, Hampshire, GU30 7JG

Several properties of the causal organism of strangles, *Streptococcus equi* subspecies *equi* (*S. equi*), would lend themselves well to eradication of the disease were our profession, along with the owners of affected horses, united and committed to best practice. Unfortunately the realism of budgetary constraints along with imperfect investigative techniques frequently leads to failure to detect horses that pose a risk of carrying the organism silently and thereby perpetuating this common and devastating disease. Undoubtedly it is the existence of silent carriers hidden among the general equine population that is of paramount importance in the epidemiology of strangles and it is their targeting which should surpass all other objectives when controlling with the disease.

Newton and colleagues (1997) suggested that one or more animals may shed *S. equi* for more than a month after the clinical signs have disappeared in more than 50% of outbreaks. Some of these cases might resolve over a few weeks although others may continue to harbour *S. equi* for years and continue to pose a risk to in contact horses (Newton et al, 1997; 2000; Sweeney et al, 1989). Thus a key aspiration following a strangles outbreak is that ***all affected and in-contact horses should be established free from infection before they are allowed to mix with other horses.***

Clinicopathologic testing for strangles comprises indirect detection via a specific serologic antibody response to the infectious agent; and direct detection of the presence of the infectious agent itself via culture or PCR. Both classes of test require very careful interpretation as the consequences of a client misunderstanding the implication of laboratory results can be substantial. This article is intended to offer a practical viewpoint of how to approach diagnostic testing and how best to use the results.

<u>BLOOD TESTING FOR STRANGLES</u>

Infection with *S. equi* tends to produce a detectable antibody response which is amenable to laboratory testing. However the use of serologic tests has important limitations which must be understood when interpreting results.

Timing of antibody response

Following initial exposure to *S. equi* serology, there will be a delay before detectable seroconversion which is probably around 2 weeks. Therefore when negative serologic results are obtained from a horse that might have been *recently* exposed to *S. equi* then it is wise to obtain a follow-up sample approximately 2 to 3 weeks later. A further interpretive difficulty is that a positive serologic result is indicative of

exposure at some time or times within the previous 6 months and not necessarily indicative of current active infection or carriage.

Diagnostic accuracy of serologic test results

There are essentially 3 *S. equi* antigenic peptides that have been exploited in commercially available serologic tests comprising the full *S. equi* M protein (SeM), the N-terminal of SEQ2190 ("Antigen A") and the N-terminal of SeM (Antigen C). Antibodies to antigens A and C are examined in a combination assay developed at the Animal Health Trust. The tests were recently compared using blood samples from 89 horses known to have been infected recently with *S. equi* and a further 139 horses resident in Iceland where *S. equi* does not exist (Robinson et al, 2013).

As indicated in table 1, the SeM based ELISA is highly prone to false positive results and there appears to be little justification for continued use of this test with far better diagnostic accuracy demonstrated by the antigen A/C test. This latter test, developed by the AHT, is also now used at other laboratories including the Liphook Equine Hospital.

Practical application of serologic tests

In a clinical context it is the reliability of positive and negative test results that the practitioner needs to know and this is indicated by the positive predictive value (PPV) and negative predictive value (NPV) respectively. These figures are highly dependent on the disease prevalence in the tested population (or disease likelihood in the tested individual) and will differ depending on the clinical scenario where testing is employed. In general terms, serologic testing is likely to be used in either a low-risk screening scenario or a high-risk investigative scenario.

a) Low-risk screening

Blood testing is often used in low-risk circumstances such as routine annual testing of a broodmare about to go to stud or testing of a new arrival directly from a low-risk premises (e.g. private home). When likelihood of disease in the tested horse(s) is low, then a negative test result will be very accurate (approximately 99% of negative tests will be correct) (Table 2). A positive test result is less reliable although still reasonably accurate. However, a positive blood test in a low-risk case merits follow up with guttural pouch examination (see below) to see if there is active infection as historical or false positive results are of no relevance.

b) High-risk investigation

Blood testing may also be used in circumstances where there is a higher probability of exposure to *S. equi*. Examples might include differentiation of exposed versus unexposed horses during a known strangles outbreak or screening of a young horse purchased from a general horse sale or market. For example, given a likelihood of disease in the tested horse(s) of 50%, then a positive test result will be highly accurate in indicating exposure to *S. equi* (table 2). However, exposure. may not always equate to *current* infection and therefore follow up testing (e.g. guttural pouch wash) is warranted to determine this. A negative test result in a high-risk case is reasonably accurate although will give false reassurance in about 1 in 16 tests and the owner of such horses should not be given absolute reassurance in this respect.

As mentioned above, follow-up testing of negative cases is advisable 2-3 weeks later if recent exposure is possible.

MICROBIOLOGIC TESTING FOR STRANGLES

Confirmation of whether or not *S. equi* organisms are actually present in the horse requires microbiologic examination. However, two important diagnostic obstacles are that the submitted samples may or may not have successfully collected *S. equi* organisms, and furthermore, the microbiologic technique may or may not detect the organism in the sample, even if present.

Culture of nasopharyngeal swabs – is it worth it?

Culture of nasopharyngeal swabs is attractive as a relatively cheap and easy technique. However, it is clear that when *S. equi* infection persists within a horse then it is most likely to be found within the guttural pouches, and may frequently not exist in the nasopharynx. Probably only between 30-45% of nasopharyngeal swabs taken from known carriers are positive on culture (Newton et al, 1997, 2000). Even taking repeated swabs does not necessarily compensate for this insensitivity as the bacterium may simply not be present in the nasopharynx. Long sequences of multiple negative nasopharyngeal swabs can be obtained despite active carriage of the infection within guttural pouches. Five out of six known *S. equi* carriers described by Newton et al (1997) would all have satisfied HBLB Code of Practice guidelines for establishing freedom from disease with up to 10 consecutive negative nasopharyngeal swab cultures. Thus ***the value of microbiologic culture of nasopharyngeal swabs is highly questionable even when repeated***.

Clearly, given financial constraints applicable to many practice situations it may well be that a client elects for culture of nasopharyngeal swabs and in such cases it is imperative that the pragmatic limitations of interpretation are clear to the client and that limited reassurance can be gained from negative results, even when repeated.

Improving the sensitivity of identifying *S. equi* in carrier horses

Further approaches that may succeed in decreasing the risk of false negative results in comparison with culture of nasopharyngeal swabs include collection of the samples from the guttural pouches, use of polymerase chain reaction and cytologic examination of such samples.

Guttural Pouch lavage

Given that the primary site for persistent *S. equi* infection is within the guttural pouches (Figure 1), it is logical to assume that a greater diagnostic sensitivity for detecting carriers will result from sampling this site. A guttural pouch wash is easy to perform as long as a few key parts to the procedure are remembered (*):

- Equipment:
  - endoscope ≥1.0 m long and ≤9 mm
  - guide wire

    - sterile catheter
    - sterile saline
- Sedate the horse
* Pass the endoscope via the *ventral* meatus (entry into the guttural pouches is very difficult via the middle meatus)
* Pass the guide wire via the biopsy channel of the endoscope and enter the guttural pouch ostium at its *most dorsal* extremity
* *Rotate* the guide wire if necessary so that it is on the *pharyngeal luminal* aspect of the endoscope (Figure 2) in order to open the ostium and allow entry (entry will not be possible with the guidewire on the *pharyngeal mural* aspect of the endoscope)
- Advance the endoscope into the guttural pouch, remove the wire and replace with sterile catheter
- Ensure the horse's head is not hanging down and instil 30 mL sterile saline into guttural pouch
- The majority of the saline will accumulate in the large medial compartment and can be aspirated from there (Figure 3)
- Place some fluid in a sterile container and some in EDTA or cytospin

Newton and colleagues (2000) compared culture of repeated simultaneous samples from the nasopharyngeal and guttural pouch in 13 known carriers. *S. equi* was successfully cultured from 59% guttural pouch washes versus 30% of nasopharyngeal samples; an approximate doubling of test sensitivity. Nevertheless the fact that 41% of carriers returned negative guttural pouch culture results highlights the fragile viability of *S. equi* in clinical samples and the need for even more sensitive diagnostic techniques.

Polymerase Chain Reaction

Diagnostic sensitivity can be further improved by using polymerase chain reaction (PCR) technology on collected samples. Webb and colleagues (2013) estimated that positive cultures of clinical samples required at least 960 viable *S. equi* whereas PCR may detect as few as 3 bacteria (G. Cordoni, unpublished data (based on *S. equi* PCR employed at Liphook Equine Hospital Laboratory)).

Two studies have compared results of PCR versus culture of diagnostic samples. Newton and colleagues (2000) found that PCR increased detection rate by approximately 21% compared with culture whereas a more recent study using a more sensitive PCR target suggested an increase in detection rate of *S. equi* by approximately 66% compared with culture (Webb et al, 2013).

Clearly selection of the probes is a crucial element of the accuracy of PCR and it cannot be assumed that all *S.equi* PCR tests are highly sensitive. It is clear that the genome of *S. equi* can vary a lot and it is unlikely that any individual PCR target will successfully detect all possible strains and subtypes of *S. equi*, especially if the PCR has not been recently designed. Continual monitoring of *S. equi* isolates and appropriate update of suitable PCR probes is essential for effective identification of strangles carriers. Both the Liphook Equine Hospital and the Animal Health Trust have recently designed new *S. equi* PCR tests targeting different and novel gene

sequences and have established high sensitivity (>93%) against a wide range of *S. equi* isolates. Use of multiple gene targets carries the significant advantage of reducing the risk of gene mutations and deletions leading to failure to detect *S. equi* organisms. (Webb et al, 2013).

There is compelling evidence in support of the use of PCR to improve *S. equi* detection rates although it is worthy of note that in one study approximately 5% of samples from known carriers resulted in successful culture of *S. equi* yet a negative PCR result (Newton et al, 2000). This may have been due to imperfect probe selection which could be improved by updated PCR design, although presence of polymerase inhibitors present in highly purulent samples would be harder to overcome. Thus although PCR has dramatically improved detection of *S. equi* in clinical samples, it should not be regarded as perfect. Neither should it be seen as a replacement for standard culture, but rather a complementary method that will enhance diagnostic power. Nevertheless even using a combination of both PCR and culture of guttural pouch washes it is still possible that carriers might be missed on rare occasions.

Cytology

If infection is present within the guttural pouch then it is to be expected that a cytologic pattern of inflammation will be present. Studies have confirmed that leucocytes are relatively sparse in the washings from normal guttural pouches (PMNs <5% total cells; Chiesa et al, 1999), although are highly prevalent within washings collected from horses with *S. equi* in their guttural pouches (Newton et al, 1997). Thus when guttural pouch washes are collected it is advisable that they are examined cytologically as well as microbiologically. Samples found to contain high numbers of neutrophils with no *S. equi* detected by culture or PCR should be regarded with additional suspicion.

Conclusions

It is advisable that serologic tests used to indicate exposure to *S. equi* are based on antigens A and C in preference to SeM serology. In all cases where a positive serology result is obtained, follow up with guttural pouch washes is important to establish whether or not any *S. equi* infection is current. Negative test results are highly reliable when taken in low-risk scenarios but caution should be exercised when interpreting a negative test result in a situation where exposure to *S. equi* is considered likely. A follow-up test 2-3 weeks later is advisable to improve reliability of establishing the true status of such high-risk individuals.

Nasopharyngeal swabs are highly unreliable for detection of *S. equi* and are very susceptible to falsely reassuring negative results. Guttural pouch lavages are vastly superior in their capacity to detect carriage of *S. equi* and should ideally be subject to a combination of microbial culture, PCR and cytologic evaluation in order to obtain the most reliable evidence for the presence or absence of current *S. equi* infection.

References

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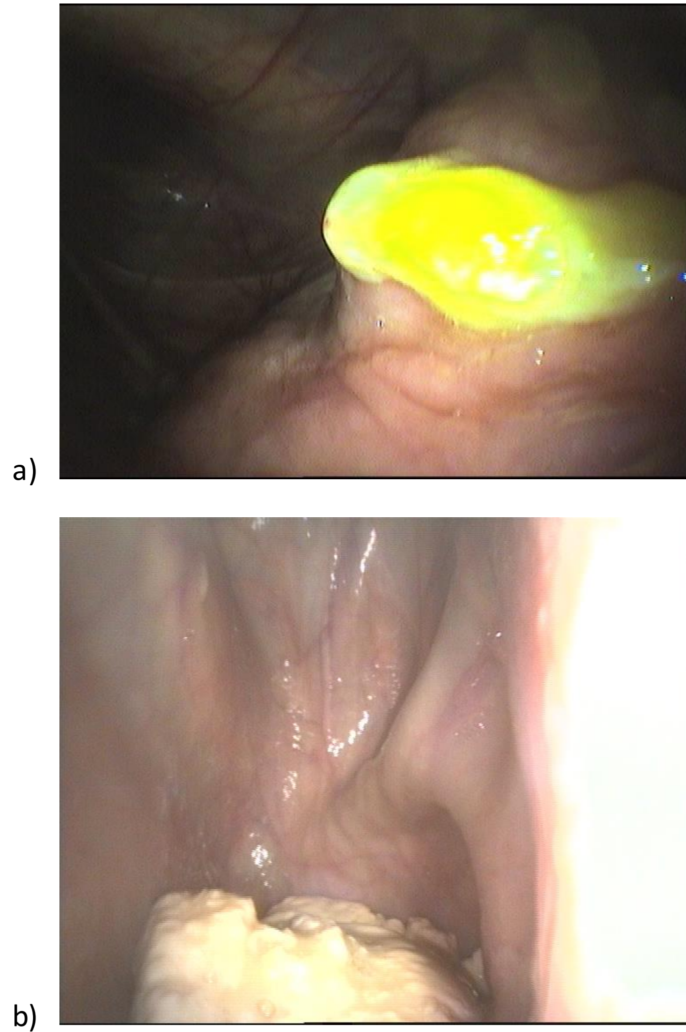
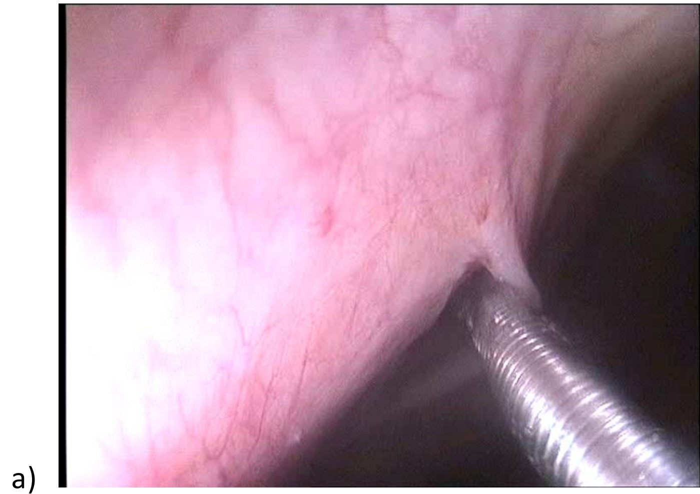


Figure 1. Endoscopic images of a) liquid pus discharging into floor of guttural pouch from retropharyngeal lymph node in a current clinical strangles case and; b) caseous chondroid material in the floor of the guttural pouch in a clinically silent *S. equi* carrier.



a)

b)

Figure 2. a) Endoscopic image showing correct approach for the right guttural pouch with wire on luminal side of endoscope; b) orientation of endoscope and wire for image a). Rotation of 180° from this orientation would be required to enter the left guttural pouch.

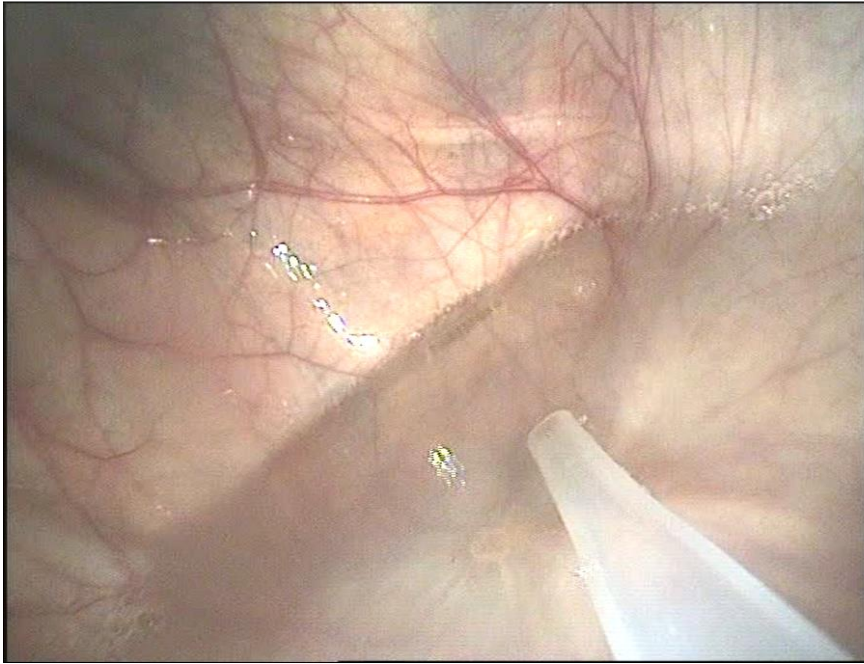


Figure 3. Collection of lavage fluid following instillation into guttural pouch.

	Infected (n=89) *(=sensitivity)*	**Unexposed (n=139)** *(=1-specificity)*
Antigen A or C	**83** *(93%)*	**1** *(1%)*
SeM	**80** *(90%)*	**32** *(23%)*

Table 1. Numbers and percentages of positive test results obtained using serologic tests against against antigens A or C and SeM using blood samples from 89 recently infected horses and 139 unexposed horses (data from Robinson et al, 2013).

	Positive result correct? (PPV)	**Negative result correct? (NPV)**
LOW RISK (10% disease likelihood)	**93.5%**	**99.3%**
HIGH-RISK (50% disease likelihood)	**99.2%**	**93.6%**

Table 2. Illustration of reliability of serologic test results based on a low-risk and high-risk scenario (data extrapolated from Robinson et al, 2013).

TEST RESULT		**SUGGESTED ADVICE/RESPONSE**
	BLOOD TEST (ANTIGEN A OR C SEROLOGY)	
Positive blood test in low-risk case	○	Guttural pouch lavage (culture, PCR, cytology) to see if there is current infection
Positive blood test in high-risk case	○	Guttural pouch lavage (culture, PCR, cytology) to see if there is current infection
Negative blood test in low-risk case	○	Offer reassurance of very low likelihood of exposure
Negative blood test in high-risk case	○	Offer caution that approximately 1 in 15 exposed horses will test negative. Consider further testing.
	CULTURE	
Positive nasopharyngeal swab	○	Confirms active *S. equi* infection
Negative nasopharyngeal swab	○	*S. equi* infection still perfectly possible (approx.55-70% of carriers test negative)
Positive guttural pouch lavage	○	Confirms active *S. equi* infection
Negative guttural pouch lavage	○	*S. equi* infection still possible (approx. 40% of carriers test negative)
	PCR	
Positive nasopharyngeal swab	○	Confirms presence of *S. equi* and probable active infection
Negative nasopharyngeal swab	○	*S. equi* infection still perfectly possible
Positive guttural pouch lavage	○	Confirms presence of *S. equi* and probable active infection
Negative guttural pouch lavage	○	*S. equi* infection unlikely but possible*
	CYTOLOGY	
Inflammatory guttural pouch lavage	○	Infection of pouch likely which may be caused by *S. equi*
Non-inflammatory guttural pouch lavage	○	*S. equi* infection unlikely but possible

*Table 4. Outline of test interpretations. (*reliability very dependent on precise test and primers used).*