A review of the methods for the detection of anthelmintic resistance.

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Received: 03.04.2002 Accepted: 18.06.2002

Abstract: Anthelmintic Resistance (AR) has been increasingly important during the last years as a consequence of the broad use of anthelmintics. Today AR is recognized as a problem worldwide involving the main anthelmintic families. Either trichostrongylid nematodes or *Fasciola hepatica* can present natural AR if environmental conditions are appropriate. In this paper we present a review of all available (standardized or not) techniques for detection of AR with their advantages and drawbacks. Although most of the methods described in this paper lack of sensibility, they are easy to develop. The complementary use of some of them along with their use on suspect farms would allow for an increase of sensitivity.
Key words: Anthelmintic resistance, Diagnosis, Gastrointestinal parasites.

Resumen: La importancia de la Resistencia Antihelmíntica (RA) se ha incrementado en los últimos años debido al amplio uso de los antihelmínticos. En la actualidad RA se considera un problema a escala mundial afectando a las principales familias de antihelmínticos. Se ha observado que cuando las condiciones son adecuadas, la RA puede aparecer tanto en tricostrongílidos como en *Fasciola hepatica*. En este artículo se presentan de forma resumida las principales técnicas (estandarizadas o no) para la detección de RA, junto con las ventajas e inconvenientes de cada una de ellas. Aunque la mayoría de los métodos descritos adolecen de suficiente sensibilidad, muchos son fáciles de desarrollar. El uso complementario de varias de ellas junto con su aplicación en explotaciones sospechosas permitiría incrementar su sensibilidad.
Palabras clave: Resistencia antihelmíntica, Diagnóstico, Parásitos gastrointestinales.

1. Introduction

Gastrointestinal parasite infections (GPI) are of utmost importance in animal production systems. Their impact on animal production is cause of important economic losses. GPI are effectively controlled by use of anthelmintics so far, however their broad use has resulted in the development of resistance.

Parasite resistance appears when there is a greater frequency of individuals within a population able to tolerate doses of a drug than in a normal population of the same species (Prichard *et al.*, 1980). Resistance develops either through a mutation or, more frequently, it is considered a pre-adaptative phenomenon, i.e. genes associated with resistance are already within the population before anthelmintic treatment is carried out; resistance is thus inherited (Jackson *et al.*, 2000).

At present, nematode Anthelmintic Resistance (AR) is becoming a problem worldwide, involving the main anthelmintic families (Van Wyk y Malan, 1988; Jackson *et al.*, 1992; Echevarria *et al.*, 1996; Hong *et al.*, 1996; Requejo-Fernández *et al.*,1997; Chartier *et al.*, 1998). In addition to trichostrongylid resistance, *Fasciola hepatica* resistance to triclabendazole has been also described (Mitchell *et al.*, 1998). Some figures on AR prevalence in small ruminants in the world are shown in Table 1.

The presence of AR depends upon factors associated with the host, the parasite, type of anthelmintic, animal management and climatic characteristics, thus increasing the difficulties for the establishment of preventive measures, which should vary according to the animal production systems (Jackson *et al.*, 2000). The wide range of factors involved, the troubles to develop new drugs and the lack of reversion to susceptibility of resistant strains (Jackson *et al.*, 1998) make AR detection of paramount importance. AR should be thus identified as soon as possible under field conditions.

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Table 1. Prevalence of anthelmintic resistance reported in small-ruminant flocks from different countries*.

Country	Percentage of resistance				
	BZs	ML	LEV	RFX	CLOS
South Africa	79	73	23	89	-
Paraguay	70	67	47	-	-
Uruguay	61	1	29	-	-
Brasil	68	7	19	-	20
Argentina	37	2	8	-	-
France	83	-	11	-	-
UK	15-44	-	-	-	-
Spain	15	10	34	-	-

Key: BZs: benzimidazoles; ML: macrocyclid lactones; LEV: levamisole; RFX: rafoxanide; CLOS: closantel.
* Sources: Jackson and Coop, 2000; Álvarez-Sánchez *et al.*, 2001.

Several AR detection techniques have been described so far, some other are still under research. In this review we present all available (standardised or not) techniques with their advantages and drawbacks.

2. *In vivo* methods

2.1. Faecal egg count reduction test (FECRT)

The FECRT has been the most recommended method so far (Waller, 1986; Jackson *et al.*, 2000), being broadly utilised either for field or research studies (Coles *et al.*, 1992; Wood *et al.*, 1995). This test is easy to perform, suitable for ruminants, horses and pigs as well as for all types of anthelmintic. In addition, it can be carried out on any species of nematodes in which eggs are shed in the faeces (Coles *et al.*, 1992).

FECRT assesses the anthelmintic efficacy of a given compound by comparing worm egg counts from animals before and after treatment. This test has been fully standardised, which has allowed for its broad use. Resistance is present when two criteria are met: 1) the percentage reduction in egg count is less than 95% and, 2) the lower limit for its 95% confidence interval is equal or below 90%. Resistance is suspected when only one of these criteria is met (Coles *et al.*, 1992). This interpretation is based on the use of the arithmetic mean, which is easier to calculate and gives better estimates of the worm egg output than the geometric mean recommended by other authors (Dagolla *et al.*, 1997).

For benzimidazoles (BZ) post-treatment egg count should be done 10-14 days after anthelmintic is administered (dpt) as anthelmintic treatment is able to temporarily suppress eggs laying without killing adult nematodes. In addition, using this period of time allows for a good correlation between the number of faecal eggs and the adult population (Coles *et al.*, 1992). In contrast, if levamisole (LEV) is used, post-treatment egg count should be carried out at day 7, as susceptible histotropic phases of the parasites could survive yielding thus a false-positive result (Grimshaw *et al.*, 1996). Some macrocyclid lactones (ML) resistant species such as *Teladorsagia circumcincta* also can temporarily suppress egg laying after treatment, yielding a false negative result at 10-14 dpt. All these aspects have to be taken into account before deciding when second sampling must be carried out.

The most important FECRT characteristic is its lack of sensibility, particularly under field conditions where mixed infections take place (McKenna, 1996; 1997a, b). This technique is not able to detect AR if the proportion of resistant worms is less than 25%, thus detecting flock resistance when it is already established (Martin *et al.*, 1989).

Despite this limitation, FECRT is one of the most suitable techniques for detection of resistance in field studies as neither specialized personnel, nor sophisticated equipment or expensive resources are needed. In addition, it can be carried out on farm without culling animals in a 10 to 14-days period. The development of coprocultures both before and after treatment is recommended as well in order to properly evaluate the results.

2.2. Controlled efficacy test (CET)

The CET is the most reliable test to assess AR against any type of anthelmintic. It is based on the quantification of the gastrointestinal nematode burden after sacrificing animals previously treated with anthelmintic (Johansen, 1989). CET is also useful to

evaluate the anthelmintic efficacy at different parasite development phases through the slaughtering of animals at different times after infection. This test must be compulsorily done before the registration of a new drug (Wood *et al.*, 1995).

CET has shown a good correlation with other type of tests such as FECRT or other *in vitro* test (Presidente, 1985), being thus used to verify the results obtained by them. It is very sensible when proper anthelmintic doses are employed (Johansen and Waller, 1989), but expensive to perform in terms of labour requirements and animal usage (Boersema, 1983), which prevents its use for routinely detection of AR.

3. *In vitro* methods

3.1. Egg hatch assay (EHA)

EHA is the generic name for a series of assays developed to detect BZ resistance. They are based on the ovicide effect of BZs and the ability of eggs from resistant strains to embryonate and hatch at higher BZ-concentrations than susceptible strains (Le Jambre, 1976; Coles and Simpkins, 1977).

This assay consists in the incubation of a known number of undeveloped eggs in serial concentrations of the anthelmintic. After 48 hours at 23°C, incubation is stopped (by adding two drops of iodine) and the percentage of egg hatching estimated for each dilution. After correcting for natural mortality, a dose-response line can be plotted against drug concentration. Data transformation (i.e. log-probit) is usually necessary in order to obtain the linear regression from which the ED_{50} (egg death 50- the concentration of anthelmintic required to kill 50% of the eggs) is calculated. ED_{50} greater than 0.1 mg/ml (ppm) is associated with AR (Figure 1). Owing to the lack of solubility of most of BZs, EHA dilutions are usually performed with thiabendazol (Johansen, 1989).

Inhibition of egg development can only be seen at initial phases, as sensitivity to BZs decreases as embryonation proceeds, therefore requiring fresh eggs to properly perform the technique. To solve this

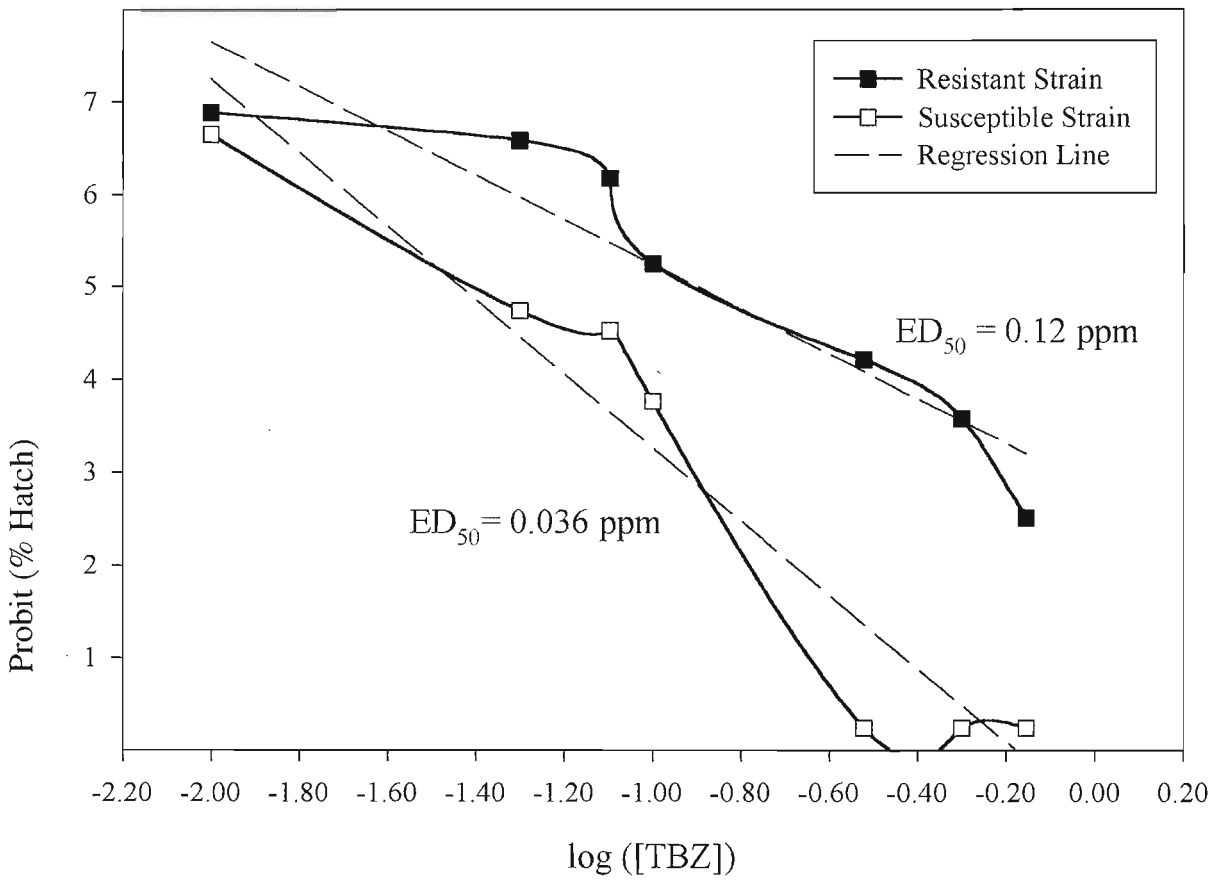

Fig. 1. Egg hatch assay (EHA) results for susceptible and resistant strains of gastrointestinal parasites from a field study on prevalence of anthelmintic resistance in small-ruminant flocks from the province of León, 2000 (Álvarez *et al.*, 2001).

problem, samples of faeces can be stored at 4°C up to 72 hours (Smith-Buijs and Borgsteede, 1986) or kept under anaerobic conditions up to 7 days (Hunt and Taylor, 1989), the latter method being the most recommended. A full description on how to storage eggs under anaerobic conditions is shown by Coles *et al.* (1992).

As FECRT, EHA lacks of sensibility, low levels of resistance (below 25%) being hardly detected (Martin *et al.*, 1989). Sensitivity seems to be affected by time of infection (Kerboeuf and Hubert, 1987; Scott *et al.*, 1990). ED_{50} would follow a parabolic pattern as time from infection increases (Borgsteede and Couwenberg, 1987; Maingi, 1991; Varady *et al.*, 1995), thus making difficult its use for field samples. An increase of test sensibility can be obtained if embryonated eggs are also considered (Johansen, 1989). When ED_{50} is slightly over 0.1 mg/ml it is advised to develop complementary tests, such as FECRT, to confirm AR.

Despite of the shortcomings described, the EHA is the method of choice to detect resistance against BZs in sheep, goats and horses (Whitlock *et al.*, 1980; Presidente, 1985; Coles *et al.*, 1992; Ihler and Bjorn, 1996; Requejo-Fernández *et al.*, 1997). It is rapid and easy to perform, and cheaper than FECRT. In addition, it correlates well with other available methods (Varady and Corba, 1999), which makes EHA a good tool for the detection of AR in field studies.

3.2. Egg hatch paralysis assay (EHPA)

The EHPA is a modification of the previous EHA in order to be used for levamisole (LEV) and morantel tartrate (MT) (Dobson *et al.*, 1986). Eggs are incubated as in EHA but the addition of the anthelmintic is done just before hatching. After 6 hours of incubation with the anthelmintic, plates are read and an ED_{50} calculated. As in the EHA, variations of ED_{50} have been observed depending on the time of infection (Varady and Corba, 1999).

EHPA presents the same disadvantages than EHA, but in this case a precise knowledge of the incubation periods is needed, which makes this technique inadequate for routine detection of AR (Johansen, 1989; Varady and Corba, 1999).

3.3. Larval development assay (LDA)

The basis of the LDA is the incubation of nematode eggs to third stage larvae in the presence of different anthelmintic concentrations. Incubation can be carried out either on liquid or solid (agar) nutritive medium.

This method is used for detection of AR against the main anthelmintic families. Also for this test, variations of LD_{50} (larval 50% death) have been observed depending on the time of infection, particularly when macrocyclid lactones (ML) are used. Absence of LD_{50} differences have been observed between ML-susceptible and ML-resistant strains when day of infection was unknown (Gill *et al.*, 1995; Amarante *et al.*, 1997). However LDA sensitivity for ML can be increase when ivermectine is replaced by avermectine-B_2 (Gill *et al.*, 1995; Amarante *et al.*, 1997). In general, the highest LD_{50} values are reached 50 to 60 days post infection for all anthelmintics.

LDA does not need of fresh eggs (Hubert and Kerboeuf, 1992), being considered a good test for AR studies or as complement of other *in vivo* and *in vitro* methods (Coles and Simpkins, 1996).

3.4. Larval motility assay (LMA)

In this case the goal of the LMA is to estimate the percentage of paralysed infective third stage larvae when incubated in a serial dilution of anthelmintic, after which a dose-response line can be plotted. As no threshold has been established yet, results must be compare to well-known susceptible strains. LMA was first used to assess resistance to LEV and MT (Martin and Le Jambre, 1979), and further developed for IVM (Gill *et al.*, 1991; d'Assonville *et al.*, 1996) and closantel (Rothwell and Sangster, 1993) on different nutritive medium (liquid, agar, enriched medium, etc.).

Initially, contradictory results were obtained for LEV because of the possibility of paralysis reversion and the lack of standardization of the observation period (Boersema, 1983). However, paralysis reversion was only observed when high concentrations of LEV (>200 ppm) were used (Geerts *et al.* 1989). The technique was adequate when a range of concentrations of 1-20 ppm and 37.5-600 ppm were employed for LEV and MT respectively, regardless the observation time (24, 48 or 72 hours).

Gill *et al.* (1991) and d'Assonville *et al.* (1996) reported a LMA for macrocyclid lactones (ML) using different nutritive medium. They observed significant differences between susceptible and resistant *Haemonchus contortus* strains. Also in this case avermectin-B_2 presented more sensitivity for detection of ML-resistance.

A modification of the LMA of Martin and Le Jambre (1979) has been used for detection of BZs-resistance. This method is based on the use of eserine (an inhibitor of the acetylcholin-esterase). The lower amounts of acetylcholin-esterase in susceptible strains

may increase binding of eserine and thus favoring paralysis of the nematodes.

LMA has been also developed for the detection of closantel-resistance. This technique was able to detect strains from 2 to 10 times more resistant (Rothwell and Sangster, 1993). So far, this is the only *in vitro* assay that works for detection of closantel-resistance.

The LMA has been shown useful to assess the ability of sheep gastrointestinal mucus to inhibit the L_3 migration. Gastrointestinal mucus from resistant ewes yielded a higher inhibition of L_3 motility than that from susceptible ewes (Rabel *et al.*, 1994).

The major weaknesses of the LMA seems to be associated with the subjective interpretation of the readings and its lack of standardization for some anthelmintics (Johansen, 1989). The main strength of LMA lies on the use of L_3, which lets both the easy performance of the technique and the identification of the resistant species in field studies.

Good correlations have been observed between LMA and other *in vitro* assays such as EHA and LDA (Rabel *et al.*, 1994; Varady and Corba,1999).

3.5. Larval feeding inhibition assay (LFIA)

LFIA is currently under research (Jackson and Coop, 2000; Álvarez-Sánchez *et al.*, in press). This assay consists in the study of the reduction of food ingestion by first stage larvae (L_1) incubated in serial dilutions of an anthelmintic plus *Escherichia coli* labelled with a fluorescent compound (fluorescein 5-isothiocyanate) as a nutritive medium. This methodology has its origin in a preliminary study described for adults by Geary *et al.* (1993), and is based on the estimation of the percentage of larvae fed for each dilution by examination of the larvae's intestine fluorescence. The Dose of Larval Feeding Inhibition 50 -$DLFI_{50}$- (i.e. the concentration of anthelmintic required to inhibit the ingestion in 50% of the L_1) is further calculated. Resistant strains would present higher LFI_{50} as they continuing eating even at lower dilutions of the drug.

This method has been tested for two families of anthelmintics so far: macrocyclid lactones (ML) and imidazothiazoles (IMZ). ML inhibit larval motility and development as well as pharyngeal pumping, the two latter are affected by 10 to 100-fold lower doses than those required to inhibit the larval motility. Thus the inhibition of food intake and larval development would be mainly a consequence of the flaccid paralysis of the pharynx (Gill *et al.*, 1995; Martin, 1996; Kotze, 1998). For IMZ, however, it is the result of a generalised muscular spastic paralysis of muscles (Sangster, 1996).

LFIA was able to discriminate resistant from susceptible strains either for ML or IMZ (Álvarez-Sánchez *et al.*, in press). $DLFI_{50}$ higher than 0.001 mg/ml would be likely associated with resistance to LM. For IMZ the differences observed were smaller than that observed for LM, being the resistance factor estimated of about 1.5.

These preliminary results show that LFIA could be a new technique for the early detection of anthelmintic resistance. It is easier and faster to perform than LDA and presents more uniform results than LMA. However some further research is required in order to standardise it.

3.6. Tubulin binding assay (TBA)

This technique is based on the affinity of BZs to parasite tubulin. TBA measures the binding of tritiated benzimidazole carbamate to this protein. Tubulin from BZ-resistant strains binds in a lesser extent with BZs than from BZ-susceptible strains (Lacey and Snowdon, 1988).

This method has been fully standardised allowing for comparisons among laboratories. It is sensitive and rapid if parasite stuff have been properly set up (Johansen, 1989). Either eggs, L_3 or adult parasites are suitable for its development. Two major drawbacks can be underlined: the need for a big amount of parasites (100,000 L_3) and the requirement of special laboratory material to work with radioactive isotopes.

It is described a good correlation between this test and EHA, LDA and CET (Johansen and Waller, 1989; Scott and Bogan, 1990).

3.7. Adult development test (ADT)

The culture of L_3 on a nutritive medium to adult nematodes has been other of the *in vitro* methods assayed (Stringfellow, 1988; Small and Coles, 1993). Differences in the development of susceptible and resistant strains of *Haemonchus contortus* to BZs have been described (Stringfellow, 1988). Similar results were also observed by Small and Coles (1993) for BZs, but not for LM and closantel.

The complexity of the culture techniques and the need of a period of waiting of around 21 days (time for reaching parasite maturity) make this method unsuitable for routine testing of AR.

3.8. Esterase activity test (EAT)

Tricostrongylid BZ-resistant strains present higher levels of non-specific esterase activity. Based on this characteristic, a colorimetric test was developed to detect resistance (Sutherland, 1989).

Despite of this characteristic, the difficulties interpreting subtle colour changes and their association with different levels of resistance prevent its use for field studies. In addition, enzymatic activity modifies as temperature changes avoiding its use as laboratory routine test as well.

3.9. Molecular techniques

Molecular techniques are of growing importance in the study of nematode AR. As molecular mechanisms of anthelmintic resistance vary for each anthelmintic family, different techniques must be set up for each drug.

In case of BZs, for which their target is the b-tubulin, resistance is mainly linked to alterations in the gene encoding for this protein (Roos *et al.*, 1990; Geary *et al.*, 1992; Kwa *et al.*, 1993a; Beech *et al.*, 1994; Lubega *et al.*, 1994; Grant and Mascord, 1996). A mutation in the amino acid 200 of the isotype 1 b-tubulin gene was identified for the main GI parasite species (*Haemonchus contortus, Teladorsagia circumcincta* y *Trichostrongylus colubriformis*) (Kwa *et al.*, 1993b, 1994, 1995; Elard *et al.*, 1996; Elard and Humbert, 1999). The mutation consists in the replacement of a phenylalanine (Phe) by a tyrosine (Tyr) at that residue. An allele-specific Polimerase Chain Reaction (PCR) was first developed to determine the genotype (Phe/Phe, Phe/Tyr, Tyr/Tyr) of adults of *T. circumcincta* on position 200 (Humbert and Elard, 1997; Elard *et al.*, 1999). Further, this method was modified to be used on L_3 and identify parasite species as well (Silvestre and Humbert, 2000).

Levamisole resistance is thought to be associated with a reduction in the number of nicotinic acetylcholine receptors or a change in their binding characteristics (Sangster *et al.*, 1988). A mutation in a transmembrane region of *lev*-1 gene, that appear to be a structural subunit of a five-subunit ACH-gated channel, changes the channel from cationic to anionic and renders *Caenorhabditis elegans* insensitive to levamisole (Fleming *et al.*, 1997). In addition, levamisole resistance has been related to the presence of low-affinity receptors in *H. contortus* (Sangster *et al.*, 1998a). However other studies suggest that levamisole resistance depends on multiple genes (Sangster *et al.*, 1998b) and that could be linked to a recessive autosomal trait (Dobson *et al.*, 1996), showing the lack of knowledge on the molecular mechanisms of levamisol-resistance.

Blackhall *et al.* (1998a) found the first gene associated with ML resistance in *H. contortus*. This gene encodes a p-glycoprotein (PGP-A) which was linked to ML resistance, suggesting thus that this gene could act as marker of resistance.

Other genes that could be involved on ML resistance are those that encode glutamate-gated chloride channels (GluCl) (Backhall *et al.*, 1998b). Part of the action of ML appears to be the potentiation of glutamate-gated opening of the GluCl channels, thus a component of the ML-resistance could be a higher level of glutamate reuptake in the vicinity of the GluCl receptors.

All these molecular tests are still under research, being those detecting BZs resistance the most developed. Further work is required to characterize the function of all these genes on the mechanisms of AR. In addition, the anthelmintic specificity of these techniques and its high cost are limiting factors for their use as routine tests.

4. Conclusions

Nematode AR is becoming a problem worldwide. The study of the presence of AR must be considered within the animal health schemes developed for each farm. The best control method is its early detection on farm. Although most of the methods described in this paper lack of sensibility, they are easy to develop. The complementary use of some of them along with their use on suspect farms would allow for an increase of sensitivity.

To detect suspect farms it is advisable to monitor the efficiency of anthelmintics after each treatment. This is a easy task as only the sampling of a random sample of animals before and after the anthelmintic treatment is needed. If the percentage of egg reduction is smaller than 95% an in-depth study of AR should be done to establish the presence of AR.

The simplest method for field studies is the FECRT. An example of how to estimate the percentage of egg reduction with its 95% confidence intervals is described in Coles *et al.* (1992). If a negative result is observed on a suspect farm, some other *in vitro* methods should be used as well.

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