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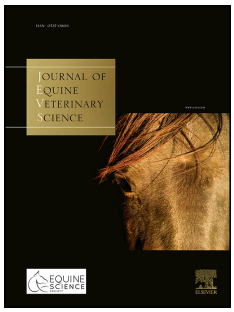

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Evaluation of locally injected *Mycobacterium* cell wall fraction in horses with sarcoids

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Abstract

A reformulation of *Mycobacterium* cell wall fraction immunotherapeutic can be used to successfully treat sarcoids in horses. Sarcoids are reported to be the most common equine skin tumors with tumor type and location influencing the choice of treatment. Wide surgical excision is curative for many tumors, but may not always be feasible. Previous studies have reported sarcoid regression after injection with mycobacterial cell wall immunotherapeutics. A new formulation of the *Mycobacterium phlei* cell wall fraction immunostimulant (Immunocidin® Equine) was utilized to treat cutaneous tumors in horses. Equids with skin tumors diagnosed as sarcoids were enrolled in the study. Sarcoids were injected at the initial visit with Immunocidin® Equine and subsequently at approximately 2-week intervals. Of 17 cases, 9 cases were completely resolved at the end of the study period evaluation or at time of final follow up

(52.9%). Three cases were reported as improved (smaller), but not resolved (17.6%). Three cases were discontinued from the study as the respective masses were growing larger or not resolving (17.6%). One case (5.8%) with two masses had resolution of one mass, whereas the other tumor had a small regrowth 5 months after the last treatment. One case (5.8%) was lost to follow up. All cases had mild to moderate swelling of the injection site, and some cases had discharge after the second, third, or fourth injections. No serious systemic side effects or complications were encountered during the study.

1. Introduction

Equids (horses, mules, donkeys, zebras) can be afflicted with various types of skin tumors. Up to 30% of cases presented for equine dermatologic disease are afflicted with cutaneous neoplasia [1,2]. In a large survey of submissions from 2 laboratories over 10 years, neoplastic disease was present in 65% of submitted samples [1]. Sarcoids are the most common type of skin tumor [1]. Many treatments have been used for sarcoids and other skin tumors including local topical and injectable therapies; response to treatment is varied and may differ depending on tumor type, tumor stage, prior treatment and location [3,6-14]. Some tumors appear to be cured with complete surgical removal [4,5,6], but wide surgical removal is not always feasible. Sarcoids often occur on the head, and in other locations that may make surgical removal difficult [6]. In addition, the variable appearance and characteristics of sarcoids can make it hard to determine prognosis [6]. No treatment for sarcoids appears to be efficacious in all cases.

Cell wall skeletons purified from bacteria have been used for many years for both human and veterinary anticancer treatments [15-20]. Attenuated *Mycobacterium bovis* strain bacille

Calmette-Guérin (BCG), and extracts from other *Mycobacterium* species, *Corynebacterium* species and other bacteria have been used as anticancer agents [18]. These immunotherapies have been found to be very effective in inducing cytokine synthesis by immune cells and activation of immune cells, as well as inducing apoptosis of cancer cells [16,18]. Side effects from bacterial cell wall products can occur and include edema, pain, tissue necrosis and discharge [14,19-22]. Previous studies report equine sarcoid regression after injection with mycobacterial cell wall products [14,19-22].

For this study, a newer formulation of a *Mycobacterium* cell wall fraction with a purportedly wider safety margin [23,24] was utilized to treat cutaneous tumors in horses. Regressin® (Bioniche Animal Health, Ontario, Canada) was an early anticancer immunotherapeutic used in animals. Immunocidin® Equine (Novavive Inc., Napanee, Ontario, Canada) is a reformulation of the original Regressin. Both products are based on the same *Mycobacterium phlei* organism. Regressin was a veterinary biologic product regulated as a poorly defined and variable sized mycobacterial cell wall extract (MCWE) and was an extract of the cell wall and other elements from *M. phlei*. Immunocidin is a veterinary biologic product regulated as a defined mycobacterium cell wall fraction (MCWF), and is purified fragments of *M. phlei* cell wall. The carrier for Regressin was phosphate buffered saline (PBS) in a 2% mineral oil emulsion; Immunocidin is formulated with *M. phlei* fractions in squalane droplets (2%) in a PBS carrier. Squalane is the fully hydrogenated form of squalene, an organic compound that is the precursor of cholesterols and steroid hormones [25].

Our hypothesis was that treatment with Immunocidin would reduce tumor mass after repeated local injection of the tumor.

2. Materials and Methods

2.1. Study Design

The study was approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University. Client-owned equids that presented to the authors' hospital for evaluation of cutaneous masses were enrolled in the study. Cases were prospectively enrolled in this open-label clinical trial by recruitment of cases presenting for skin tumor treatment. Owners were informed of the study aims, procedures, risks and aftercare and indicated their consent for inclusion in this study by signing a consent form approved by IACUC. Inclusion criteria were horses with single or multiple masses presumptively diagnosed as sarcoids during physical exam, and which did not exceed 5 cm diameter. In case where multiple tumors were present, the largest mass was not to exceed 5cm in diameter. The size restriction was chosen to limit variability for this trial. Signalment and tumor location were recorded. A physical examination was used to assess general health. Only 1-3 of the largest tumors were injected while small adjunct masses or ones in close proximity to the largest injected tumor were left untreated. Initial work-up included history, physical examination, tumor biopsy, tumor measurement and photography. A 4 or 6 mm punch biopsy instrument was used to obtain a sample of the mass during the initial examination. Biopsies were evaluated by a boarded pathologist to establish a histopathologic diagnosis.

2.2. Injection

Volume of injection was calculated with the following formula suggested by the product manufacturer: Length (cm) x Width (cm) = cm^2 x 0.78 (used for 'rounding' the corners of the L

x W multiple) = calculated cm^2 x 0.5 ml = X ml (total volume to be injected). Minimum recommended dose is 0.5 ml/ cm^2 tumor surface area. Injections were performed with a 22-gauge needle (or 20 gauge if a dense fibrous sarcoid) into the mass in a grid pattern. Needles were placed approximately 0.8 cm apart and a total of 2/3 the calculated dose was injected into the tumor using the grid pattern. One third of the calculated dose was also injected under the tumor.

2.3. Monitoring and Additional Treatment

Horses received examination and treatments every 14 days. If no visible tumor were present at recheck, then an additional 14 day recheck was recommended and no treatment was performed at that time. If the tumor were still visible, an additional treatment was performed at that time. If no change in mass size or consistency was seen after 4 injections, or if the tumor were growing larger during treatment, then treatment was discontinued and other methods such as surgical removal or chemotherapy were recommended to treat the tumor. Tumors were photographed and measured at each visit prior to re-injection. Any adverse events were recorded, including but not limited to swelling, discharge, tissue necrosis, pain, scarring. Owners were notified in advance of treatment of possible side effects. Tumors were considered resolved or regressed if the original mass(es) were gone at last evaluation or at final follow up. Tumor resolution was defined as the skin appeared normal or had a scar (but no visible tumor tissue).

2.4. Statistical analysis

Tumor size was calculated at each time point using the following formula: Length (cm) x Width (cm) = cm^2. Data sets were tested for normality with the Shapiro–Wilk test and found to be not

normally distributed. Differences in in tumor size between time points were analyzed by the one-way Friedman repeated measures analysis of variance on ranks with Student -Newman-Keuls post-hoc test for multiple comparisons. Data were presented as median and interquartiles or ranges. Commercial software (GraphPad Software, San Diego, California) was used for statistical analyses with significance established at $P < 0.05$.

3. Results

3.1. Horses

Twenty-one equids ranging in age from 4 to 23 years (median 11 years), were initially enrolled in the study. Histopathologic diagnosis was reported as sarcoid for 16 cases; 1 case was diagnosed as a peripheral nerve sheath tumor, which is a histologic diagnosis often given for sarcoids (see discussion). Histologically, 4 of the cutaneous lesions were diagnosed as other masses, despite appearing to be sarcoids based on examination. One case was diagnosed as a "suspect cyst or neoplasm", and three cases as dermatitis. Only the cases diagnosed as sarcoid or peripheral nerve sheath tumor were statistically analyzed for comparison. Breeds or species included in the final analysis included 10 Quarter Horses, 3 Thoroughbreds, one each of Missouri Fox Trotter, Paint, draft cross, and warmblood. A summary of results is provided in **Table 1**.

3.2. Case Outcome

Complete data sets were available for analysis for 15 horses on days 0, day 14, 28 and 42. If tumor was resolved, a value of 0 was given for the size measurement. Differences in the tumor size between time points are presented in **Figure 1**. The median tumor size was significantly

smaller on day 42 and 28 compared to day 14 ($P<0.05$). Of 17 cases, 9 (52.9%) cases were completely resolved at the end of the study period evaluation or at the time of final follow up (see examples in **Figures 2 and 3**). Three cases were reported to have improved (were smaller), but did not resolve (17.6%). Three cases were discontinued from the study as the respective masses were growing larger or not resolving (17.6%). One of those cases had no appreciable change in size during treatment and was slightly larger 1 month after the last treatment, but has remained static since that time. Another case had a noticeable increase of size of the tumor and treatments were discontinued after the third injection; the tumor was surgically removed and the base of the tumor treated with imiquimod. The third case was not responding to treatment and discontinued, but additional treatments or surgical procedures of the mass are unknown. One case (5.8%) with two masses had resolution of one mass, whereas the other tumor had a small regrowth 5 months after the last treatment; the regrowth was treated with CO_2 laser ablation and imiquimod after which both masses have remained resolved. One case (5.8%) was completely lost to follow up and outcome could not be determined despite repeated attempts to contact owner and referring veterinarian.

3.3. Treatments and Side Effects

The number of injections completed ranged from 2-5 injections (median 4). Volume injected during treatments varied based on size of tumor according to the manufacturer's recommended formula. Average injection volume was 4.6 milliliters (ml), range 0.5-14 ml). All cases had swelling at the site of injection and the immediate surrounding area following injection, typically starting at about 24-48 hours post injection. Swelling gradually resolved, but sometimes took several weeks and up to 3 months to completely return to normal. Swelling was typically firm in

the area just under and around the injection site. Pitting edema was evident in many cases around or ventral to the injection site. Six cases had discharge of purulent appearing material from the injection site and/or mild sloughing of superficial tissues (**Figure 4**). Discharge occurred after the 2nd, 3rd or 4th injection (not after the first injection). All owners were instructed to administer NSAIDS if needed in the event that significant swelling or soreness occurred. Injection was well tolerated in most cases; some horses required sedation or twitch application for the injections.

3.4. Follow up

Final follow up was available for 16 cases, and time from last treatment to final follow up averaged 9 months (ranged 4 - 17 months). Follow up with the owner and/or referring veterinarian was completed by phone and/or email communication. In some cases, follow up was completed during examination by the authors at a recheck examination or during a visit to our clinic for another reason. If the owner or referring veterinarian reported the outcome, they were asked to report presence of any remaining mass after treatment, regrowth of new masses, and the presence of scar tissue. When possible, photos were obtained to corroborate owner or referring veterinarian follow up information given, as seen in **Figure 5**. One case did not show up for appointments for treatments and recheck examinations after the second treatment, but the owner did later respond to follow up communication and reported that the mass had resolved and not returned. One case was completely lost to follow up. The owner did not show up for their second appointment and did not return further communications, thus no final outcome could be obtained for this cases. Three cases were withdrawn from the study due to treatment failure; masses were getting larger despite treatment.

4. Discussion

Our findings indicate that Immunocidin® Equine was effective in 52.9% of the cases enrolled that had a known outcome, after 2-5 injections. The treatment was safe, and although the side effect of swelling occurred in every case, swelling of the injection site was transient and resolved with no other treatment than NSAID administration. Regression rates in these cases are similar to previous reports of sarcoid treatment with BCG [5,14,26-28]. Although not statistically significant (P=0.08), there was an increase in the median tumor size 14 days after the initial treatment. This transient increase in tumor size may be explained with inflammation from the injection.

Mycobacteria and their cell wall components have been used to treat a variety of tumors in both human and veterinary patients [6,14,22-29]. Treatments prepared from mycobacterial components are typically described or defined as non-specific immunotherapies; their ability to stimulate the immune system has been well studied [18]. Mycobacterial cell complexes induce cytokine synthesis and apoptosis, and activate immune effector cells [16-18]. T-lymphocytes are important elements of the anti-tumor response to mycobacterial cell wall-based treatments [17]. Toll-like receptors, which are expressed on dendritic cells and macrophages, recognize many microbial structures. Mycobacterial antigens stimulate these cells through specific toll-like receptors, and rapid activation of the innate immune system occurs [30]. In some studies, mycobacterial cell complexes have also been shown to inhibit cellular proliferation of cancer cell lines [16]. Mycobacterial cell complexes have been reported in some human studies to have a better toxicity profile than BCG [23,24]. No explicit statements of pretreatment for anaphylaxis

were reported in these studies, but some practitioners report pre-treating with nonsteroidal anti-inflammatories before the use of BCG. The authors did not pre-treat with anti-inflammatory medications during this study, but did advise owners to administer NSAIDS if needed in the event that significant swelling or soreness occurred. Our study supports a low rate of serious adverse events with the use of MCWE, although we recognize our study population is small.

Not all masses in this study were diagnosed by histopathology as sarcoids. Because biopsies were taken at the time of the first treatment, and histopathology was not completed until later, some masses were diagnosed as sarcoids based on clinical exam, but subsequent histopathologic diagnosis for 2 cases were determined to be eosinophilic dermatitis and 'suspect cyst or neoplasm' for one case. Although these cases were not included in the final analysis, 2 of these 3 cases resolved after treatment, indicating that this product might be a useful treatment for masses other than sarcoids. It is possible, however, these masses may have resolved without treatment. Diagnosis of masses that appear to be sarcoids may not be confirmed with histopathology for several reasons; as their diagnosis by histopathology has challenges and limitations [28]. Other authors have noted that the histologic appearance of sarcoid fibroblasts are not different from normal fibroblasts, and that diagnoses of fibroma, fibroscarcoma, and nerve sheath tumors are sometimes made in cases that later were discovered to be sarcoids [28]. The authors did include the one cased diagnosed as peripheral nerve sheath tumor in the final analysis for this reason. Additionally, because the treatment in this study was an injection and the authors sought to preserve tumor mass to monitor response to this treatment, the biopsies taken were small (4-6 mm) punch biopsy samples instead of larger excisional biopsy tissue samples. Such small samples may have provided an inadequate amounts of tissue, especially if

inadvertently taken from an area of the mass with only associated inflammatory changes, or without changes characteristic of sarcoids.

It has been reported previously that different types of sarcoids respond differently to therapy, including immunotherapy such as BCG [22,28]. Our analysis of percent change in tumor size when compared by location or type of tumor did not reveal any statistically significant differences. These results are shown in **Figures 6** and **7**. A larger sample size would be necessary to further discriminate among the different sarcoid types and locations with respect to treatment response.

Three cases were improved or smaller, but not completely resolved. It is possible with further injections, that resolution may have been achieved. While these three masses were reported to be smaller than at initial examination and quiescent at the time of follow up, owners either chose to not pursue further treatment at all, or chose to pursue a different treatment at a later date. One difficulty in conducting clinical trials is continued client participation. In this study, participants were asked to bring their horses for examinations and treatments every 2 weeks. Some clients found the effort to trailer horses to our clinic so frequently burdensome, especially if they traveled longer distances. Conducting the examinations and treatments on farm may have increased compliance, but the authors in this study were not primary ambulatory practitioners, and many cases that were enrolled were outside of our ambulatory radius. Evaluation of the final outcome can also be difficult during clinical trials if the patient is not re-presented to the clinic. If owners or referring veterinarians are contacted and no response obtained, final outcome is not known and these cases cannot be included in final analysis; thus, there is loss of data. In this

study, most owners were relatively easy to contact, responded, and were willing to answer follow up questions.

5. Conclusions

This reformulation of mycobacterial cell wall fraction for use in horses, Immunocidin® Equine, appears to be a safe and effective treatment for some sarcoids. Though they were transient and resolved with either no treatment or with the use of NSAIDs, owners and veterinarians should be aware of the likelihood of post injection complications including swelling and possible discharge from the site of injection. The mycobacterial cell wall product studied appears to be viable and reasonably effective option for the treatment of sarcoids in equine patients, which remains an important cause of pathology in horses, and a sometimes difficult condition to treat.

Financial disclosure

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354 **Table 1.**
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Age (years)	Breed	Histopathologic diagnosis	Location	Mass Type	Day 0	Day 14	Day 28	Day 42	% Change in tumor size Day 0 to Day 42	Outcome at final follow up	Duration - last treatment to final follow up
18	Paint	Peripheral nerve sheath tumor	R neck	occult	4.1 cm x 2.8 cm	3.6 cm x 1.3 cm	-	0	-100.00	Resolved	16 mos
12	QH	Sarcoid	R periocular	nodular	1.7 cm x 2.0 cm	2.7 cm x 3.2 cm	appeared resolved	0	-100.00	Resolved	14 mos
5	Draft cross	Sarcoid	L TMJ area	occult and nodular	2.5 cm x 2.5 cm	3.4 cm x 1.9 cm	2.5 cm x 1.6 cm	0	-100.00	One mass resolved. One resolved then regrowth 5 mos after last tx.	12 mos
12	QH	Sarcoid	R sheath and inguinal area	fibroblastic	4.0 cm x 2.5 cm	-	-	-		Tx failure; masses getting larger	Withdrawn from study before 2nd visit
16	TB	Sarcoid	LH heel bulb	fibroblastic	4.3 cm x 5.3 cm	3.8 cm x 7.2 cm	3.8 cm x 5.5 cm	3.8 cm x 5.5 cm	-8.29	Improved. Smaller but not gone.	7 mos
13	QH	Sarcoid	Chest, periocular, elbow, shoulder	nodular and verrucose	2.5 cm x 3.0 cm	4.3 cm x 4.3 cm	2.7 cm x 3.1 cm	2.0 cm x 3.1 cm	17.33	Resolved	17 mos
11	QH	Sarcoid	L prepuce	mixed	3.1 cm x 3.2 cm	3.1 cm x 4.8 cm	3.5 cm x 3.2 cm	4.0 cm x 2.0 cm	-19.35	Resolved	9 mos
5	M Fox Trotter	Sarcoid	R cheek	fibroblastic	3.6 cm x 4.8 cm	3.0 cm x 4.2 cm	2.8 cm x 3.6 cm	3.0 cm x 3.5 cm	-39.24	Improved. Smaller but not gone.	8 mos
7	QH	Sarcoid	L chest	verrucose	4.5 cm x 3.6 cm	4.0 cm x 3.5 cm	4.0 cm x 3.5 cm	4.0 cm x 4.1 cm	1.23	Improved. Smaller but not gone	5 mos
11	QH	Sarcoid	LH pastern	fibroblastic	1.3 cm x 1.5 cm	1.5 cm x 1.5 cm	1.5 cm x 2.0 cm	2.1 cm x 2.5 cm	5.25	Tx failure; mass getting larger.	Withdrawn from study at 4th visit
5	QH	Sarcoid	L neck	nodular	0.5 cm x 0.6 cm	0.9 cm x 1.0 cm	0.76 cm x 1.14 cm	0	-100.00	Resolved	12 mos
4	QH	Sarcoid	R neck, L ear	nodular and occult	4.5 cm x 5.2 cm	5.3 cm x 7.0 cm	4.0 cm x 7.0 cm	4.0 cm x 7.5 cm	28.21	Resolved	4 mos
13	WB	Sarcoid	R ear	fibroblastic	3.2 cm x 3.6 cm	-	-	-		Lost to follow up	Lost to follow up
7	TB	Sarcoid	R periocular	nodular and occult	4.0 cm x 5.0 cm	3.4 cm x 4.0 cm	3.4 cm x 4.0 cm	3.8 cm x 3.8 cm	-27.8	Resolved	5 mos
7	TB	Sarcoid	L shoulder	occult	2.5 cm x 2.3 cm	2.0 cm x 1.5 cm	sloughed off	0	-100.00	Resolved	4 mos
12	QH	Sarcoid	R chest	fibroblastic	3.0 cm x 2.2 cm	2.8 cm x 2.5 cm	2.3 cm x 2.2 cm	2.6 cm x 2.5 cm	-1.52	Tx failure. Mass slightly larger at 7 mos post tx.	Withdrawn from study at 4th visit
11	QH	Sarcoid	RH dorsal cannon	mixed	2.0 cm x 2.3 cm	1.9 cm x 1.9 cm	1.5 cm x 2.4 cm	1.1 cm x 2.2 cm	-47.39	Resolved	6 mos

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Figure Legends

Figure 1 Differences in tumor sizes (centimeters squared) (n=15) between time points (day). Asterisk denotes statistically significant difference ($P<0.05$). Median and interquartiles.

Figure 2 Sarcoid on the prepuce in an 11-year-old Quarterhorse gelding before (A), during (B), and after (C) treatment.

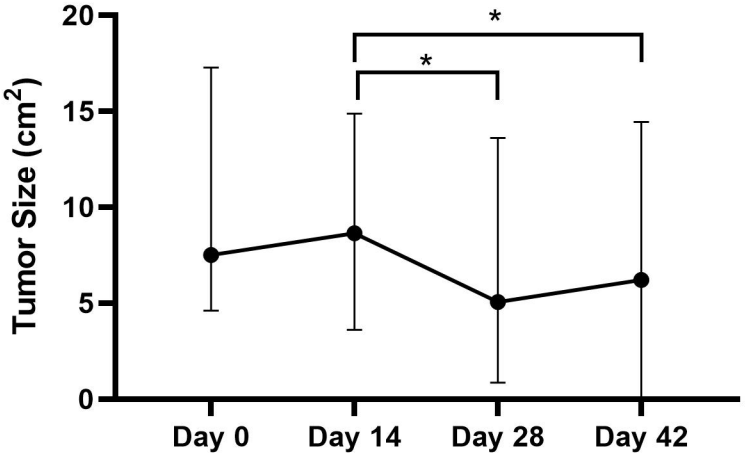
Figure 3 Periorbital sarcoid in a 7-year-old Thoroughbred gelding before (A), during (B), and after (C) treatment.

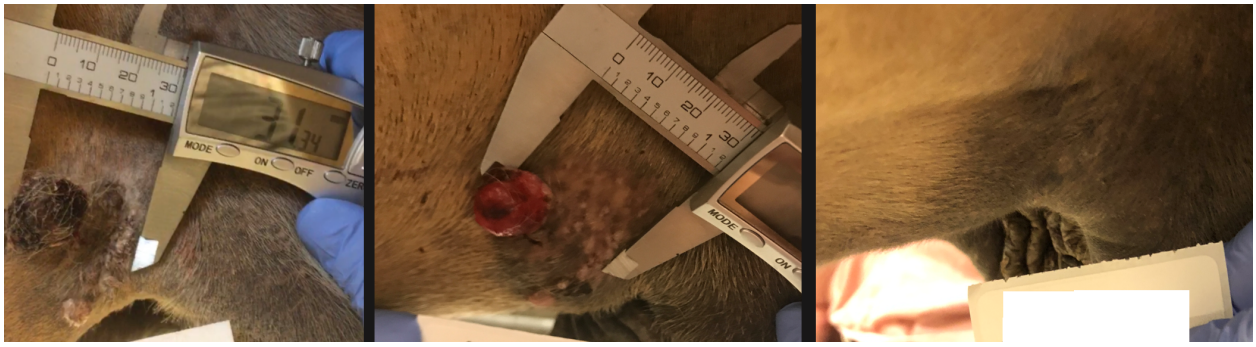
Figure 4 Sarcoid on the neck in a 4-year-old Quarterhorse gelding exhibiting purulent appearing material from the injection site and sloughing of superficial tissues.

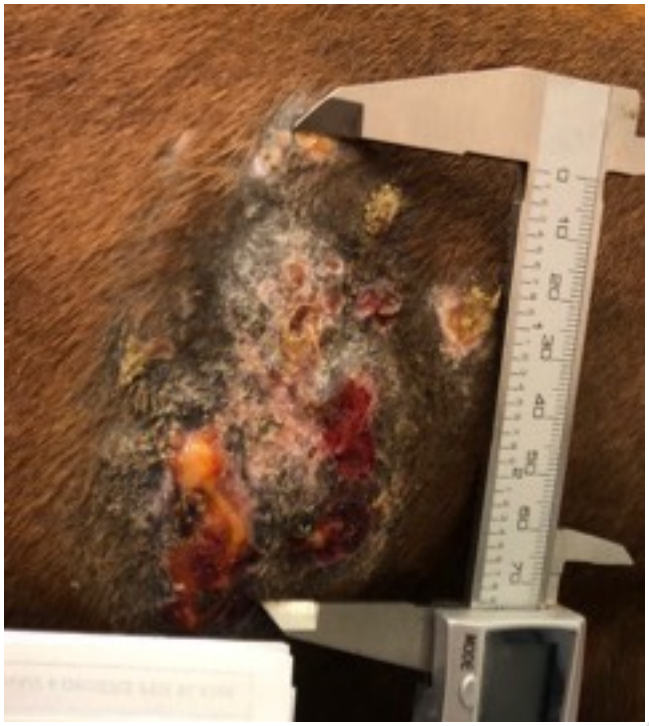
Figure 5 The same horse from Figure 4 after resolution and healing of the treatment site, 5 months after the last treatment. A scar is present at the site of treatment.

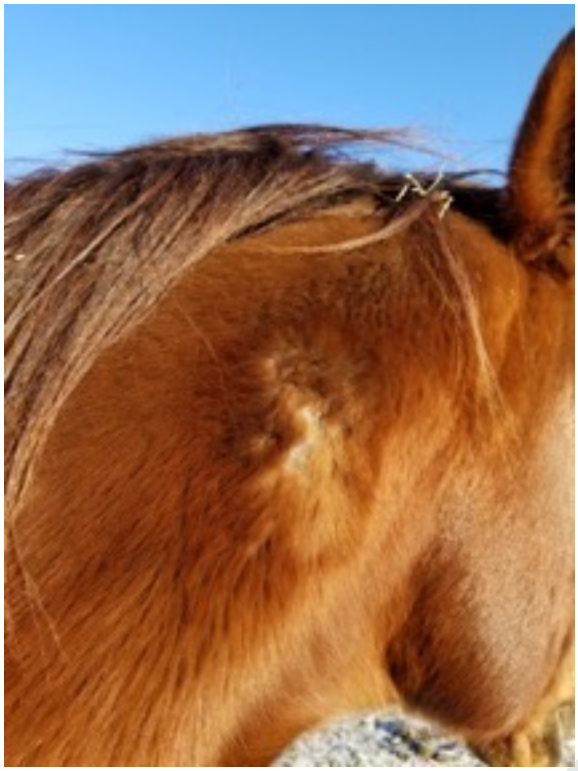
Figure 6 Median change in tumor size (percent change) from day 0 to day 42 by location.

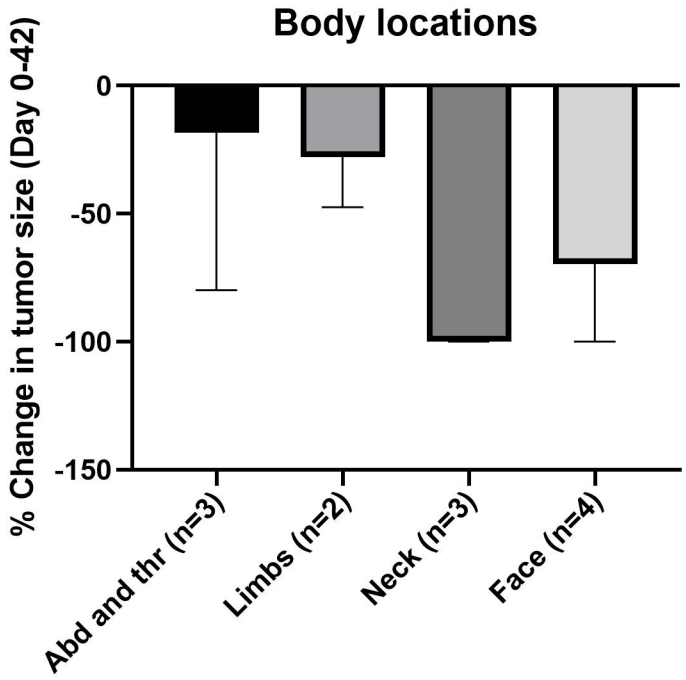
Figure 7 Median change in tumor size (percent change) from day 0 to day 42 by tumor type.



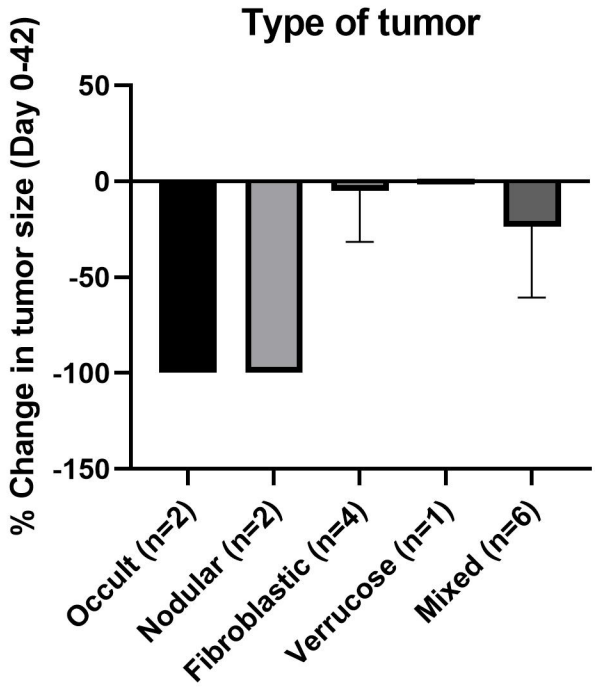








Abd and thr - abdomen and thorax



Highlights

Mycobacterial cell wall fractions can be used to successfully treat some sarcoids in horses.

Mycobacterial immunotherapies induce cytokine synthesis, apoptosis, activate immune cells.

Minimal side effects of swelling and discharge are common with mycobacterial cell wall immunotherapies.

Ethical Statement

This study was approved by the Institutional Animal Care and Use Committee (IACUC). Client-owned equids that presented to the authors' hospital for evaluation and treatment of cutaneous masses were enrolled in the study. Owners were informed of the study aims, procedures, risks and aftercare and indicated their consent for inclusion in this study by signing a consent form approved by IACUC.

Conflict of Interest Statement

Immunocidin® Equine product, and funding to cover the cost of histopathologic evaluation of tissues, the cost of examination visits, and publication costs were provided to the authors' institution by Novavive, Inc.