PEER REVIEWED

Case Study - Attempted Treatment of a Cow with *Mycobacterium paratuberculosis* Enterocolitis

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Abstract

A Holstein-Friesian dairy cow naturally infected with *Mycobacterium paratuberculosis*, and suffering from enterocolitis, was treated orally with a combination of rifampin, pyrazinamide and streptomycin (30, 50 and 25 mg/kg/day, respectively) for seven months. Clinical and hematological evaluations were regularly performed beginning on the day of admission, extending throughout treatment and continuing until four months after therapy was terminated. Therapy was associated with normalization of hematological and serum biochemical parameters, and a 440 lb (200 kg) weight gain. This positive effect was observed during the entire treatment period and persisted for nearly four months after the end of therapy. Microscopic examination of Ziehl-Neelsen stained fecal samples was negative for *M. paratuberculosis* from the second day, and remained negative until the fourth month after therapy was completed. Fecal cultures remained positive for *M. paratuberculosis* throughout the observation period. The cow was harvested four months after cessation of therapy. These observations suggest that chemotherapy results in improvement in clinical signs, and might induce a transient reduction of *Mycobacterium paratuberculosis* shedding. It did not eradicate the infection or eliminate shedding of the organism.

Résumé

Une vache laitière Holstein-Friesian infectée naturellement avec *Mycobacterium paratuberculosis* a été traitée oralement avec une combinaison de rifampin, pyrazinamide et streptomycine (30, 50 et 25 mg/kg/jour, respectivement) durant une période de sept mois. Des évaluations cliniques et hématologiques ont été faites régulièrement à partir du jour d'admission et se sont prolongées pour la durée du traitement et les quatre mois suivant la fin du régime thérapeutique. La thérapie était associée à la normalisation des paramètres hématologiques et biochimiques du sérum et à un gain de 440 livres (200 kg). Cet effet bénéfique était observé sur toute la durée du traitement et persista pendant la période de quatre mois suivant la fin du régime thérapeutique. Des examens microscopiques d'échantillons fécaux avec la coloration de Ziehl-Neelsen étaient négatifs pour la présence de *M. paratuberculosis* du second jour jusqu'à quatre mois après la thérapie. Des cultures fécales restaient positives pour la présence de *M. paratuberculosis* pendant toute la période d'observation. La vache était récolté quatre mois suivant la fin de la thérapie. Ces observations suggèrent que la chimiothérapie produit une amélioration des signes cliniques et pourrait aussi promouvoir une réduction temporaire de l'excrétion fécale de *M. paratuberculosis*. Le traitement n'a pas éliminé l'infection ni l'excrétion fécale de l'organisme.

Introduction

Many *in vitro* and *in vivo* studies have been performed during the last thirty years to evaluate the efficacy of different drugs, administered alone or in combination, for the control of *Mycobacterium paratuberculosis* infection, or Johne's Disease, in ruminants.[3,4,5,8,10,12,13,14,16,17,18,19,20,22,24]

The efficacy of a combination of rifampin, pyrazinamide and streptomycin for treatment of *M. paratuberculosis* infection was previously evaluated in our studies on experimentally infected calves.[3,4,5] This drug combination has been successfully employed for the treatment of tuberculosis in humans.[6] The different actions of these drugs, i.e., in the intracellular (rifampin and pyrazinamide) and extracellular (rifampin and streptomycin) compartments, results in a synergistic effect. However, antibacterial drugs have never been shown to stop fecal shedding of mycobacterial organisms in studies performed on naturally infected animals.[19,24]

In this study, the therapeutic efficacy of the combination of rifampin, pyrazinamide and streptomycin was evaluated in a naturally infected dairy cow suffering from clinical Johne's disease.

Clinical Case

A six-year old female Holstein-Friesian dairy cow suffering from clinical paratuberculosis was admitted to the Institute of Special Pathology and Veterinary Medicine of the University of Milan. She was referred from an infected herd that was participating in a voluntary paratuberculosis eradication program. The cow had calved four months earlier, but was not rebred because she was scheduled to be culled and slaughtered as part of the disease eradication plan.

During the monitoring period, the experimental protocol included daily clinical examination and regular collection of blood for hematological, serum biochemical, immunological and bacteriological evaluation.

Hematology, Serum Biochemistry and Immunology

Blood samples were collected at admission, on day 15 and once monthly for the entire hospitalization period. Hematologic examination was done on the day of blood collection. Serum was divided into two aliquots; the first was used to determine (within 4 hours from collection) biochemical parameters, such as albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, creatinine, alkaline phosphatase (ALP), γ-glutamil-transpeptidase (γ-GT), glucose, lactic-dehydrogenase (LDH), potassium, sodium and total proteins. The second aliquot was immediately frozen at -112°F (-80°C) and stored for serum protein electrophoresis[a] and IgG, IgM and IgA immunoglobulin titration.[b]

Urinalysis

Urine was collected by catheter on the day of admission, on day 15 and once monthly during the entire hospital stay. Gross, microscopic and chemical examinations[c] were performed.

Rumen Fluid Analysis

Rumen fluid was collected on the day of admission with a Dirksen's guidable probe and evaluated by physical and microscopic examination, as well as Methylene Blue reduction test.

Fecal Examination

A fecal sample collected from the cow on the day of admission was examined by flotation for the presence of fecal nematode eggs.

Direct microscopic examination and culture of feces for *M. paratuberculosis* were performed weekly. Samples were collected directly from the rectum using sterile gloves. Microscopical examinations were performed on freshly prepared fecal smears that were air-dried and stained with Ziehl-Neelsen stain. For fecal culture, 10 g of feces from each sample were decontaminated with 0.75% hexadecylpyridinium chloride solution, and then cultured on Herrold egg-yolk medium, as described by Belletti *et al.*[2]

Therapeutic Procedure

Following admission of the cow to the hospital, fluid and electrolyte imbalances were corrected using Lactated Ringer's Solution. An analgesic- antispasmodic drug (propil-N-fenotiazina fumarate BID for 4-5 days) was used to control colic pains. By day 15, the cow had improved clinically, and hematologic and serum biochemical values had returned to more normal levels, except for persistent hypoproteinemia and hypoalbuminemia (Table 1).

Once the cow had stabilized (day 15), anti-infectious treatment was initiated, which consisted of a combination of rifampin, pyrazinamide and streptomycin administered orally once daily at 30, 50 and 25 mg/kg, respectively. The antimicrobial combination was administered for seven months.

Clinical and Laboratory Outcomes

At admission, the cow presented with the following clinical signs; depression, tenesmus, muscular atro-

[a]Performed on agarose gel using Titan Gel, High Resolution Procedure kit, available from Helena Laboratories, Beaumont, TX, USA.

[b]Performed by Radial Immunodiffusion Assay (RID) using a commercially available kit available from VMRD Inc., Pullman, WA, USA.

[c]Combur test, Boehring Mannheim, Germany.

Table 1. Hematological and serum biochemical values.

Blood parameters	1	2
PCV (28-36%)	45	32
RBC (5-8 M/mm^3)	9.93	6.97
Hb (8-12 g/dl)	15.7	11
WBC (4-10 K/mm^3)	13.1	11.3
Glucose (2.5-3.3 mmol/l)	13	3.46
Creatinine (< 110 µmol/l)	132	106
Na (135-150 mmol/l)	119.7	128
K (4-5 mmol/l)	2.83	3.54
Total bilirubin (< 8.5 µmol/l)	5.13	15.39
ALT (7-14 U/l)	15	13
AST (< 40 U/l)	91	97
ALP (< 150 U/l)	89	123
γ-GT (< 20 U/l)	6.5	7.5
LDH (500-1500 U/l)	910	1040
Total protein (6-8 g/dl)	5.9	5.1
Albumin (3-4 g/dl)	1.97	1.6
A/G (0.5-0.55)	0.5	0.45

Blood parameters 1 = at admission
Blood parameters 2 = after 15 days

phy, dehydration, severe watery diarrhea, mucosal congestion, absence of rumination, oliguria, agalactia and no fever. Abdominal muscles were contracted. Rumen motility was decreased, but intestinal borborygmi were increased in both number and intensity. Hematologic and serum biochemical results showed hemoconcentration, hyperglycemia, hypokalemia, and hypoproteinemia related to hypoalbuminemia with a consequent marginal albumin/globulin ratio (Table 1). Hypo-γ-globulinemia was also evident.

At initial examination, urine specific gravity was 1.030, the pH was 6 and there was evidence of glycosuria (5.5 mmol/l). Examination of ruminal fluid revealed several abnormalities; dark brown color, watery consistency, fast sedimentation rate, reduced particulate matter, delayed clearance time with the Methylene Blue reduction test, and hypo-motile flora. No gastrointestinal parasite eggs were found. A high number of acid-fast bacteria were detected in Ziehl-Neelsen stained fecal smears. Fecal culture was positive for *M. paratuberculosis* organisms.

Symptomatic therapy resulted in improvement in clinical signs within 15 days following hospitalization. This favorable physiological status was considered a necessity before starting the antimicrobial therapy.

Antimicrobial therapy was associated with noticeable clinical improvement. The cow regained her appetite, with normal rumination and ruminal motility, and normal feces. Lactation had ceased, therefore we were unable to assess any effect on milk production. Serum protein levels (Table 2) and high-resolution electrophoresis values returned to normal.[15,21] Likewise, IgG, IgM and IgA serum immunoglobulin titers did not show relevant variations and remained within normal ranges.[15,21] Improvement was evident during the seven month treatment period and the following four months observation period. During this time, the cow gained 440 lb (200 kg).

No acid-fast bacteria were found in feces stained with Ziehl-Neelsen stain from the second day of antimicrobial therapy through the fourth month post-therapy. In contrast, fecal cultures were positive during the entire experimental period.

Four months after the cessation of therapy, the cow's general condition remained good and serum IgG, IgM and IgA levels were normal, however, total protein and albumin values began to decline (Table 2). The reappearance of diarrhea with the finding of acid-fast bacilli on microscopic fecal examination confirmed that the cow had relapsed. For this reason, the cow was humanely euthanized.

At necropsy, a chronic proliferative enterocolitis with lymphadenopathy, consistent with paratubercular infection, was observed. Microscopic examination of hematoxylin and eosin stained sections showed chronic enteritis characterized by a lymphocytic infiltrate and giant cells in the mucosal *tunica propria* and in the intestinal submucosa. Ziehl-Neelsen stained sections were positive for acid-fast bacteria in the cytoplasm of macrophages and giant cells.

Discussion

Hypoproteinemia and hypoalbuminemia present prior to therapy are typical of a paratubercular protein-losing enteropathy. Low initial γ-globulinemia levels suggest a diminished immune response, contributing to the severity of the infection. Some authors have reported a polyclonal gammopathy in both the pre-clinical and clinical paratuberculosis.[1,11] During antimicrobial therapy, serum immunoglobulin levels did not change significantly and remained within normal limits.[7,9] Hypoproteinemia and hypoalbuminemia four months post-therapy, and prior to the reoccurrence of diarrhea, are typical findings in clinically active paratuberculosis cases.[1,11]

Results of this study suggest that antimicrobial therapy can reduce or eliminate the clinical signs of paratuberculosis, but cannot eliminate the infection. Others have reported similar results.[19,24] During antimicrobial therapy and for a period of time in the post-therapy period, acid-fast organisms could not be detected in samples stained with Ziehl-Neelsen stain, however, fecal cultures remained positive for paratuberculosis.

Extracellular rifampin and streptomycin activity[6] is restricted largely to the intestinal lumen and thus

Table 2. Hematological and serum biochemical values during treatment and the post-therapy periods.

	Treatment period (months)							Follow up period (months)			
Blood parameters	1	2	3	4*	5	6	7	8	9	10	11
PCV (28-36%)	30	28	27	26	28	31	32	37	31	38	31
RBC (5-8 M/mm^3)	6.92	6.78	5.87	5.87	6.17	6.53	6.45	7.52	7.41	7.74	6.94
Hb (8-12 g/dl)	11.5	9.2	9.5	9.0	9.8	10.2	11	11.9	11.6	11.2	11.2
WBC (4-10 K/mm^3)	9.9	9.6	13.8	24	11.7	13.4	14.3	11.8	13.6	7.9	10.5
Glucose (2.5-3.3 mmol/l)	3.41	3.46	3.19	3.35	4.12	3.35	3.02	2.86	3.08	3.52	3.02
Creatinine (< 110 µmol/l)	97	79	88	115	79	79	79	97	106	115	97
Na (135-150 mmol/l)	138	138.6	137.4	136.2	136.3	139.4	141.5	137.5	141.7	141.3	137.5
K (4-5 mmol/l)	4.3	4.51	5.7	3.58	4.42	3.8	4.22	4.7	3.78	3.7	4.32
Total bilirubin (< 8.5 µmol/l)	13.7	13.7	6.8	3.4	3.4	3.4	3.1	3.9	3.4	3.4	14.3
ALT (7-14 U/l)	10	5	6	8	11	13	10	17	15	19	7
AST (< 40 U/l)	55	43	35	23	25	25	21	29	30	24	47
ALP (< 150 U/l)	110	131	59	67	116	80	84	78	69	57	116
γ-GT (< 20 U/l)	6.8	7	8.2	7	4	7	4	3	7	2.4	7.3
LDH (500-1500 U/l)	1068	1960	1115	738	1000	1080	935	1061	1024	930	1150
Total protein (6-8 g/dl)	6	7.2	8.2	8.4	7.8	8.7	7.7	7.8	7.9	7.6	6.1
Albumin (3-4 g/dl)	1.7	2.1	2.7	2.9	3.3	3.7	3.3	3.7	3.6	3.5	1.6
A/G (0.5-0.55)	0.39	0.41	0.49	0.52	0.6	0.74	0.75	0.9	0.8	0.8	0.36

Blood parameters 1-7 = evaluated during treatment
Blood parameters 8-11 = evaluated after treatment
*The leukocytosis observed on the fourth month coincided with an infection of the extremity.

has the potential to reduce fecal shedding of *M. paratuberculosis* organisms, but not eliminate them. The persistence of the infection might be explained by inadequate blood and tissue levels of the drugs with intracellular activity (rifampin and pyrazinamide), and/or by insufficient treatment duration. In fact, pyrazinamide is rapidly absorbed in the rumen, whereas rifampin absorption occurs primarily in the intestine.[23] In this case study, rifampin absorption could be negatively influenced by abomasal ingesta, and in particular by the pathological conditions in the intestinal mucosa, i.e., the characteristic proliferative lesions seen at necropsy and histopathological examination.

The drugs, the dosage, duration of therapy and route of administration used in this case study were similar to those employed in previous studies we conducted on experimentally infected calves. However, in our earlier studies, a prophylactic and not a curative treatment was attempted.[3,4,5] To establish whether a cure is possible, the efficacy of this treatment needs to be evaluated in naturally infected calves as well as in older diseased animals. When the infection is active, the drugs may be unable to adequately penetrate the intracellular spaces where the organism resides, thereby resisting treatment. Further research might determine whether higher doses of rifampin and pyrazinamide, which have intracellular activity, could achieve therapeutic intracellular concentrations and eradicate the infection.

Conclusions

Long-term administration of combination antimicrobials to a cow naturally infected with *M. paratuberculosis* resulted in improvement of clinical signs, more normal hematologic and serum biochemical values, but did not eliminate shedding or infection. The cow could likely serve as a reservoir for infection of younger animals on the farm. Caution should be exercised to avoid confusing clinical improvement with cure of the disease.

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Editor's Comments

Rifampin, pyrazinamde and streptomycin are not approved for use in food animals in the United States.

Meat or milk from farm animals treated with these drugs or used in research studies should not be used for human consumption.

References

1. Abate O, Tarducci A, Cagnasso A, Dotta U, Zanatta R: Studi preliminari sul quadro proteico e sul livello sierico delle IgG e IgM in bovine con infezione paratubercolare clinicamente non manifesta. *Atti SIS Vet* 46:1483-1487, 1992.
2. Belletti GL, Arrigoni N, Boni P, Invernizzi A, Sacchi R, Zanardi G: Isolamento di *Mycobacterium paratuberculosis* da feci bovine: confronto fra tre differenti terreni colturali. *Atti SIS Vet* 44: 905-908, 1990.
3. Belloli A, Belletti GL, Agosti M, Pozza O, Greppi G, Arrigoni N, Proverbio D, Avezza F: Chemioprofilassi nell'infezione sperimentale da *Mycobacterium paratuberculosis*. *Atti SIS Vet* 44: 1413-1417, 1990.
4. Belloli A, Arrigoni N, Vacirca G, Agosti M, Pozza O, Greppi G, Proverbio D: Efficacy of two associations of antituberculous drugs in calves experimentally infected with *Mycobacterium paratuberculosis*. Third Intern Coll on Paratuberculosis, 508-511, 1991.
5. Belloli A, Arrigoni N, Belletti GL, Proverbio D, Greppi G, Vacirca G: First results of paratuberculosis therapy in calves experimentally infected by intravenous route. 4th Intern Coll on Paratuberculosis, 144-146, 1994.
6. Blasi A, Donatelli L, Zanussi C: Rifampicina. Ed. Minerva, 131-138, 1978.
7. Bonizzi L, Amadori M, Melegari M, Ponti W, Ceccarelli L, Bolzani E: Characterization of some parameters of non-specific immunity in dairy cattle. *J Vet Med B* 36: 402, 1989.
8. Chiodini RJ: Bactericidal activities of various antimicrobial agents on human and animal isolates of *Mycobacterium paratuberculosis*. *Antimicrob Agents Chemother* 34: 366-367, 1989.
9. Dall'Ara P: Immunologia: Caratteristiche del Sistema Immunitario nelle diverse specie animali. In Poli G & Cocilovo A "Microbiologia e Immunologia Veterinaria". Ed. UTET, Torino. 727-760, 1996.
10. Das SK, Sinha RP, Chauan HVS: Chemoterapy of paratuberculosis in goats: streptomycin, rifampicin and levamisole *versus* streptomycin, rifampicin and dapsone. *Indian Journal Animal Science* 62: 8-13, 1992.
11. Dotta U, Abate O, Tarducci A, Cagnasso A, Zanatta R: Quadro siero-proteico nella paratubercolosi bovina. *Obiettivi e Documenti Veterinari* 3: 63-68, 1994.
12. Eidus L, Denst H: The susceptibility of *Mycobacterium paratuberculosis* (Johne's Bacillus) to chemotherapeutic agents. *Am Rev Dis* 89: 289-292, 1964.
13. Gezon HM, Bither HD, Gibbs HC: Identification and control of paratuberculosis in a large goat herd. *Am J Vet Res* 49: 1817-1823, 1988.
14. Hoffsis GF, Streeter RN, Rings DM, St Jean G: Therapy of Johne's Disease. *Bov Pract* 25: 55-58, 1990.
15. Kaneko JJ: Clinical Biochemistry of Domestic Animals. 4th ed., Academic Press, Inc. 1989.
16. Larsen AB, Vardamam TH, Groth AH: Preliminary studies on the effect of streptomycin and other agents on *Mycobacterium paratuberculosis*. *Am J Vet Res* 11: 374-377, 1950.
17. Larsen AB, Vardamam TH: The effect of viomycin 4:4' Diaminodiphenyl sulphone and other agents on *Mycobacterium paratuberculosis*. *Am J Vet Res* 13: 466-468, 1952.
18. Larsen AB, Vardamam TH: The effect of isonicotinic acid hydrazide on *Mycobacterium paratuberculosis*. *J Am Vet Med Assoc* 122: 309-310, 1953.
19. Merkal RS, Larsen AB: Clofazimine treatment of cow naturally infected with *Mycobacterium*. *Am J Vet Res* 34: 27-28, 1973.
20. Rankin JR: An attempt to prevent of Mycobacterium Johne in calves with isoniazid alone and in combination with streptomycin. *Vet Rec* 67: 1105-1107, 1955.
21. Rosenberger G: L'esame clinico del bovino. Essegivi, Piacenza. 1993.
22. St Jean G, Jernigan AD: Treatment of *Mycobacterium paratuberculosis* infection in ruminants. *Vet Clin North Am Food An Prac* 7: 793-804, 1991.
23. Villa R, Sonzogni O, Belloli A, Arrigoni S, Guarneri D, Caloni F, Carli S: Studio preliminare sulla cinetica ematica dell'associazione Rifampicina, Pirazinamide e Streptomicina nel vitellone. *Atti SIS Vet* 49: 711-712, 1995.
24. Whitlock RH, Divers T: Johne's Disease: a case of study with clofazimine therapy. *Proc Inter Coll Res On Paratuberculosis*, Ames, Iowa. 14: 231-237, 1983.