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Negri Bernardino, Pedro

de Paula, Carolina

Pereira, Ana

et al.

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VETERINARY MICROBIOLOGY - SHORT COMMUNICATION

Potential in vitro action of an adenosine analog and synergism with penicillin against *Corynebacterium pseudotuberculosis*

Pedro Negri Bernardino[1,2] · Carolina Lechinski de Paula[1] · Ana Flávia Marques Pereira[3] · Márcio Garcia Ribeiro[1] · Vasco Ariston de Carvalho Azevedo[4] · Alexandre Secorun Borges[1] · Ary Fernandes-Júnior[3] · José Paes Oliveira-Filho[1]

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Abstract

Caseous lymphadenitis is a well-known disease caused by *Corynebacterium pseudotuberculosis* affecting small ruminants with small significance to human health because of its minor zoonotic potential. In both cases, few treatment options are available and conventional antimicrobial therapy is commonly refractory due to development of pyogranulomatous reactions, bringing great interest in discovering novel therapeutics for more suitable approaches. Dideoxynucleotides presented antibacterial action against various bacteria but were never described for *C. pseudotuberculosis*. Hypothesizing the antimicrobial action of 2',3'-dideoxiadenosine (ddATP) against *C. pseudotuberculosis*, we performed for the first time an investigation of its minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in the ATCC® 19,410 strain and a well-characterized clinical isolate of *C. pseudotuberculosis*. We also assessed potential synergism with penicillin. ddATP showed a growth delay effect for *C. pseudotuberculosis* at 2 µmol/mL and a MIC and MBC of 4 µmol/mL against the ATCC® 19,410 strain, but not for the clinical strain. An antimicrobial effect was observed when using concentrations lower than the MIC of ddATP associated with penicillin for both strains tested. Our data suggest the potential of nucleotide analogs, especially adenosine, and its combination with penicillin, as a possible novel treatment for *C. pseudotuberculosis*-induced infections, and contributes with knowledge regarding alternative drugs to treat *C. pseudotuberculosis* infections.

Keywords 2',3'-Dideoxyadenosine · Caseous lymphadenitis · Bactericidal · Bacteriostatic · Small ruminant

Introduction

Corynebacterium pseudotuberculosis is a Gram-positive bacteria and facultative intracellular pleomorphic organism able to infect animals and humans, causing caseous lymphadenitis [1, 2]. Usual clinical signs consist of regional swelling and abscess formation in the lymph nodes or subcutaneous space [3, 4]. Diagnosis is through in vitro culture or inoculation in laboratory animals of caseous content of abscesses or molecular and serologic tests [5]. Vaccines have moderate effectiveness mainly due to non-followed manufacturer recommendations [6], and early removal of clinically and/or serologically positive animals from herds proved to be good prophylaxis [7, 8]. Routine treatment is based on surgical removal or drainage of abscesses and a long period of antibiotics therapy (4 to 6 weeks of penicillin or erythromycin combined with rifampin), and alternative techniques are being studied, but all methods present low efficacy or high chances of relapse [9–13]. This information shows that new efficient therapeutic methods to lower economic and genetic losses in small ruminant flocks have become of great interest.

Dideoxynucleotides (ddNTPs) differ from deoxynucleotides by a single hydrogen molecule replacing the hydroxyl in the 3' carbon of the pentose ring, becoming chain terminators during the synthesis of DNA by blocking the link of the next nucleotide into the sequence [14, 15]. Current

Responsible Editor: Luis Nero

✉ Pedro Negri Bernardino
pnberna@ucdavis.edu

[1] School of Veterinary Medicine and Animal Science, São Paulo State University (UNESP), Botucatu, Brazil

[2] School of Veterinary Medicine, Molecular Biosciences Department, University of California – Davis, Davis, USA

[3] Institute of Biosciences, São Paulo State University (UNESP), Botucatu, Brazil

[4] Genetics, Ecology and Evolution Department, Federal University of Minas Gerais, Belo Horizonte, Brazil

research aiming to repurpose existing nucleotide analog drugs that are commonly used in immunomediated conditions, neoplasia, or viral therapies identified multiple drug candidates with an in vitro antibacterial activity [16]. Early experiments on the antibacterial action of 2',3'-dideoxyadenosine (ddATP) were observed in vitro and in vivo for several bacteria [17, 18]. Recent studies found the growth inhibition potential of purine analogs in multiple commensal and pathogenic gastrointestinal bacteria [19, 20] caused by a disruption in the mechanisms of DNA replication or even inducing DNA break [21], but none of those studies included *Corynebacterium* species.

We hypothesize that ddATP presents antibacterial action against *C. pseudotuberculosis* and could be potentially used to favor the current treatment protocols recommended to caseous lymphadenitis. In this scenario, we investigated for the first time the in vitro action of ddATP against standard *C. pseudotuberculosis* biovar *ovis* culture and possible synergism between ddATP and penicillin.

Material and methods

Ethical approval statement

This study was approved on July 10, 2019 by the Institutional Animal Care and Use Committee (118/2019 – CEUA – UNESP) of São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Botucatu/SP, Brazil.

Corynebacterium pseudotuberculosis isolates

A well-characterized and sequenced strain of *C. pseudotuberculosis* (ATCC® 19,410) from the Institute of Biological Sciences of the Federal University of Minas Gerais and one strain isolated of a goat abscess at the Microbiology Laboratory of the School of Veterinary Medicine and Animal Science from UNESP, Botucatu, were used in this study. They were stocked in a glycerinated brain heart infusion (BHI) broth at – 80 °C.

ddATP and penicillin

The 2′3'-dideoxyadenosine (ddATP) was purchased from Sigma-Aldrich (Cotia, SP), diluted in MiliQ water, and stored frozen in – 20 °C, as specified by the manufacturer. The penicillin used was the commercial Aricilina® (Blau Farmacêutica S.A.; Cotia, SP), diluted in MiliQ water, and stored in 4 °C, no longer than 30 days.

Minimum inhibitory concentration and minimum bactericidal concentration of ddATP for ATCC® 19410 strain

Culture conditions in Mueller Hinton (MH) were tested and adapted from the literature [22, 23], serial dilution, and MIC and MBC procedure followed CLSI 2018 guidelines [24], and previous description of ddATP antibacterial action and its MIC values for other bacterial species was considered to establish a starting concentration [18]. In summary, initial experiments were conducted with ddATP diluted into a 16 µmol/mL solution of MH broth with 0.2% of Tween 80, followed by serial dilution to 50% of the concentration from 16 to 0.001 µmol/mL in a 96-well flat-bottom plate. Each well had an initial volume of 75 µL. The *C. pseudotuberculosis* ATCC® 19,410 colonies were incubated in BHI agar and dissolved into 0.85% saline solution until turbidity reached the 0.5 McFarland standard and further added to the MH broth with 0.2% Tween 80 for a bacterial concentration of 1.5×10^6 bacteria/mL. Seventy-five microliters of this final solution was added to each well with the ddATP (as described in the previous paragraph), reaching a final volume of 150 µL per well and making 8 µmol/mL the highest concentration of ddATP to be tested. Positive controls consisted of the same culture media volume and bacterial concentration in the well but no ddATP, and negative controls had only culture media with no bacteria. The plate was incubated at 37 °C on a shaking platform (220 rpm) in aerobic conditions for 48 h. Three evaluations were made: first, a subjective turbidity evaluation of each well; second, an optic density reading by a spectrophotometer (Epoch™ 2, BioTech®) on a 600 nm wavelength [25]; and third, a resazurin reduction test [26]. After optic density measurement, 50 µL of 0.05% resazurin was added to each well, incubated for 2 h under the same previous conditions, and observed for the color of the final solution, where pink indicates the presence of bacterial growth and blue/purple violet indicates growth inhibition. The agreement of the three results gave a consistent MIC for ddATP.

Before the resazurin test, the wells with no apparent growth by presenting low optic density and clear solution and the positive control were cultivated in a BHI agar Petri dish using calibrated handles. They were incubated at 37 °C in aerobic conditions for 48 h and then evaluated according to the colony growth. The MBC was considered the concentration that presented no growth or a significantly low colony count (no more than 10 colonies) compared to the positive control (uncountable due to proximity of colonies).

MIC and MBC of penicillin and challenged bacterium growth inhibition curve

The same procedures described above were performed using penicillin for both strains. The concentration ranged from 8 to 0.03125 µg/mL, using two-fold serial dilution.

Growth inhibition curve

Using the spectrophotometer (Epoch™ 2, BioTech®), a growth curve for the bacteria incubated with ddATP and/or penicillin was made. The plate was incubated under the same conditions in the machine, and optic density readings were performed every 2 h until 48 h of incubation.

MIC, MBC, and growth inhibition curve for the clinical isolate

The same procedures to achieve the MIC and MBC were followed as described above, but using the clinically isolated strain and a testing concentration for the ddATP of 4 µmol/mL (the MIC found in the ATCC® 19,410 strain).

Penicillin and ddATP synergism

C. pseudotuberculosis ATCC® 19,410 and clinical strain were incubated in MH broth with 0.2% Tween 80 and a mix of penicillin and ddATP at 1 µmol/mL (25% of their MIC) with 50% or 25% of the penicillin. A growth inhibition curve, as specified previously, was assembled and compared with the curves of the positive controls (bacteria cultivated alone; bacteria cultivated with each substance alone) to determine any difference. Each well with a clear medium after the 48th hour was cultivated in a BHI petri dish for another 48 h for MBC testing. A resazurin test was also performed after the first 48 h of incubation in the broth.

Results and discussion

Previous studies showed fourteen bacteria species susceptible to ddATP, ten presenting a MIC lower than 2.5 µmol/mL and four ranging from 6 to 13.5 µmol/mL [18]. Our initial findings suggest an intermediate MIC of ddATP (4 µmol/mL) for *C. pseudotuberculosis* ATCC® 19,410 strain compared to these other bacteria, besides a bactericidal action at the same concentration (MBC = 4 µmol/mL). Once growth curves were evaluated, it was possible to observe a right displacement of the curve indicating delayed bacterial growth when ddATP was at 2 µmol/mL, but complete inhibition of bacterial growth could only be observed in concentrations equal to or higher than 4 µmol/mL of ddATP (Fig. 1a). The growth curve of an *Escherichia coli* mutant challenged with ddATP also indicated bacteriostatic action in a lower concentration of ddATP (0.1 µmol/mL) and bactericidal in higher (0.3 and 1 µmol/mL) within the first 60 and 180 min, respectively [17]. Moreover, other purine analogs (azathioprine and mercaptopurine) also presented in vitro inhibitory potential that was concentration-dependent [19, 20]. Penicillin presented a MIC of 0.125 µg/mL and MBC of 0.25 µg/mL on *C. pseudotuberculosis* ATCC® 19,410. To our knowledge, no available investigation was carried out focused on purine nucleotide analogs combined with antibiotics to acquire a synergism against bacteria, despite a pyrimidine analog that has shown synergism against *Staphylococcus aureus* [27]. By using penicillin in lower concentrations than its MIC (50%) with ddATP at knowingly sub-MIC (25%), inhibition of *C. pseudotuberculosis* growth (Fig. 1b) and bactericidal action were observed. These results indicate a possible positive synergism of penicillin with a purine analog, a synergism between two classes of molecules (purine analog and beta-lactam antibiotic) unstated in the literature, to our knowledge.

Attempting to replicate these results in a clinical isolate of *C. pseudotuberculosis*, the bacteria were incubated with ddATP at a concentration of 4 µmol/mL, and subsequent two-fold dilutions presented only a right displacement of the growth curve but not total inhibition of bacterial growth (Fig. 2a). Synergism was tested using 25%

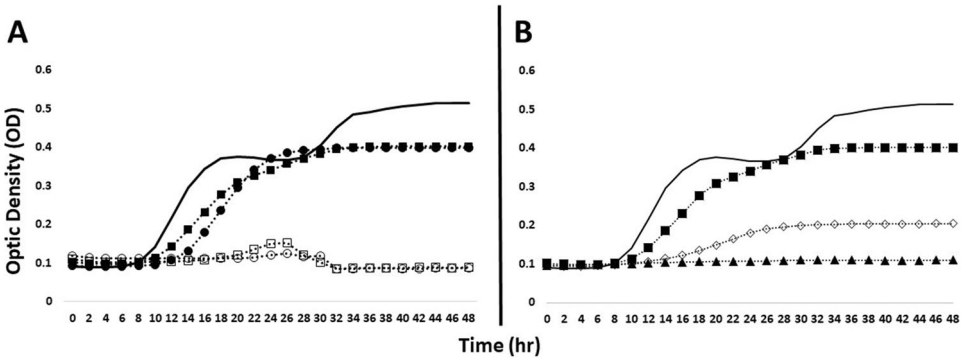

Fig. 1 Growth curve of *Corynebacterium pseudotuberculosis* ATCC 19,410 challenged with different concentrations of ddATPs (**a**) or challenged with the combinations of ddATP and penicillin in concentrations below their minimum inhibitory concentration (MIC) (**b**). Labels: (▬) positive control – no challenge; (○) 8 µmol/mL ddATP; (□) 4 µmol/mL ddATP; (●) 2 µmol/mL ddATP; (■) 1 µmol/mL ddATP; (▲) 1 µmol/mL ddATP + 50% penicillin MIC; (◊) 50% penicillin MIC

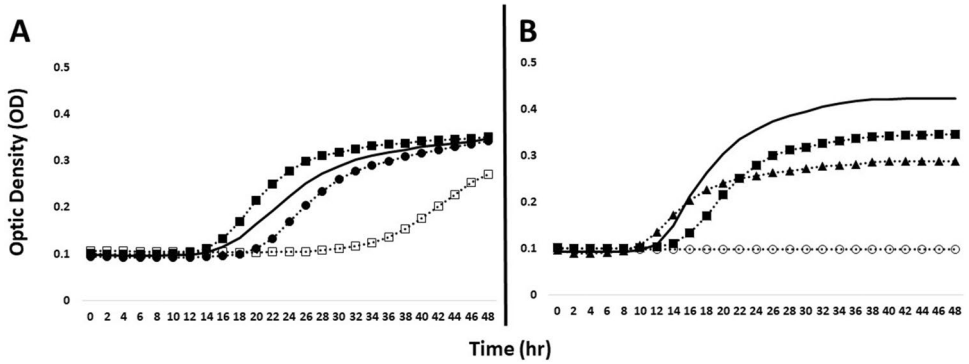

Fig. 2 Growth curve of *Corynebacterium pseudotuberculosis* clinical strain challenged with different concentrations of ddATPs (**a**) or challenged with the combinations of ddATP and penicillin in concentrations below their minimum inhibitory concentration (MIC) (**b**). Labels: (▬) positive control – no challenge; (□) 4 µmol/mL ddATP; (●) 2 µmol/mL ddATP; (■) 1 µmol/mL ddATP; (○) 1 µmol/mL ddATP + 25% penicillin MIC; (▲) 25% penicillin MIC

of penicillin MIC combined with 1 µmol/mL of ddATP (lower than 25% of its MIC that is higher than 4 µmol/mL), which inhibited bacterial growth (Fig. 2b) and had a bactericidal action demonstrated by absence of *C. pseudotuberculosis* colonies in agar BHI petri dishes. Penicillin MIC and MBC were 0.25 µg/mL. If confirmed by further studies in more significant numbers of strains of these bacteria, our data could indicate the use of purine analogs as a new therapeutic option for *C. pseudotuberculosis* infection. It is essential to state, however, that this was an important first step to testing our hypothesis and further evaluations are essential before fully considering these nucleotide analogs as new effective therapeutic methods, using larger sample sizes and studying mechanistic features, such as the ability of these compounds to penetrate abscesses walls through biofilm assays or in vivo trials.

Overall, it is known that *C. pseudotuberculosis* infection in sheep and goats presents a high rate of recurrence or failure in healing after conventional clinical and/or surgical treatment [9–12], and even human therapeutics show hardships in achieving total healing [28], probably due to development of pyogranulomatous reactions that difficult conventional antimicrobials reach therapeutic concentrations inside foci of lesions. The results, for our purpose, revealed that ddATP can disrupt *C. pseudotuberculosis* growth and have a synergistic effect with penicillin, which could be a novel alternative to therapy approaches of *C. pseudotuberculosis* infections in small ruminants.

Author contribution PNB and JPOF contributed to the conception and design of the study. PNB and CLP contributed to the samples collection. PNB, CLP, MGR, VACA, and AFJ contributed to the processing of the samples. PNB, ASB, and JPOF contributed to writing the first drafts of the manuscript. All authors contributed to manuscript revision, and read and approved the submitted version.

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Data Availability Not applicable.

Declarations

Competing interests The authors declare no competing interests.

References

1. Dorella FA, Pacheco LGC, Oliveira SC, Miyoshi A, Azevedo V (2006) *Corynebacterium pseudotuberculosis*: microbiology, biochemical properties, pathogenesis and molecular studies of virulence. Vet Res 37:201–218. https://doi.org/10.1051/vetres:2005056
2. Baird GJ, Fontaine MC (2007) *Corynebacterium pseudotuberculosis* and its role in ovine caseous lymphadenitis. J Comp Pathol 137:179–210. https://doi.org/10.1016/j.jcpa.2007.07.002
3. Bastos BL, Portela RWD, Dorella FA, Ribeiro D, Seyffert N, Castro TLP, Miyoshi A, Oliveira SC, Meyer R, Azevedo V (2012) *Corynebacterium pseudotuberculosis*: immunological responses in animal models and zoonotic potential. J Clin Cell Immunol 4:1–15. https://doi.org/10.4172/2155-9899.S4-005
4. Zavoshti FR, Khoojine ABS, Helan JA, Hassanzadeh B, Heydari AA (2012) Frequency of caseous lymphadenitis (CLA) in sheep slaughtered in an abattoir in Tabriz: comparison of bacterial culture and pathological study. Comp Clin Pathol 21:667–671. https://doi.org/10.1007/s00580-010-1154-7
5. Oreiby AF (2015) Diagnosis of caseous lymphadenitis in sheep and goat. Small Rumin Res 123:160–166. https://doi.org/10.1016/j.smallrumres.2014.11.013
6. Windsor PA, Bush RD (2016) Caseous lymphadenitis: present and near forgotten from persistent vaccination? Small Rumin Res 142:6–10. https://doi.org/10.1016/j.smallrumres.2016.03.023

7. Baird GJ, Malone FE (2010) Control of caseous lymphadenitis in six sheep flock using clinical examination and regular ELISA testing. Vet Rec 166:358–362. https://doi.org/10.1136/vr.b4806
8. Jesse FFA, Bitrus AA, Abba Y, Chung ELT, Sadiq MA, Hambali IU, Perera H, Haron AW, Lila MAM, Saharee AA, Norsidin MJ, Harith A (2013) Strategic management of a clinical case of recurrent caseous lymphadenitis in a goat farm. Res J Vet Practitioners 4:42–6. https://doi.org/10.14737/journal.rjvp/2016/4.3.42.46
9. Gezon HM, Bither HD, Hanson LA, Thompson JK (1991) Epizootic of external and internal abscesses in a large goat herd over a 16-year period. J Am Vet Med Assoc 198:257–263
10. Williamson LH (2001) Caseous lymphadenitis in small ruminants. Vet Clin N Am - Food Anim Pract 17:359–371. https://doi.org/10.1016/s0749-0720(15)30033-5
11. Olson ME, Ceri H, Morck DW, Buret AG, Read RR (2002) Biofilm bacteria: formation and comparative susceptibility to antibiotics. Can J Vet Res 66:86–92. https://doi.org/10.1016/s0749-0720(15)30033-5
12. Guimarães AS, Carmo FB, Pauletti RB, Seyffert N, Ribeiro D, Lage AP, Heinemann MB, Miyoshi A, Azevedo V, Gouveia AMG (2011) Caseous lymphadenitis: epidemiology, diagnosis and control. Inst Integr Omics Appl Biotechnol J 2:33–43
13. Sellera FP, Gargano RG, Libera AMMPD, Benesi FJ, Azedo MR, Sá RLM, Ribeiro MS, Baptista MS, Pogliani FC (2016) Antimicrobial photodynamic therapy for caseous lymphadenitis abscesses in sheep: report of ten cases. Photodiagn Photodyn Ther 13:120–122. https://doi.org/10.1016/j.pdpdt.2015.12.006
14. Toji L, Cohen SS (1969) The enzymatic termination of polydeoxynucleotides by 2',3'-dideoxyadenosine triphosphate. Proc Natl Acad Sc USA 63:871–877. https://doi.org/10.1073/pnas.63.3.871
15. Tsesmetzis N, Paulin CBJ, Rudd SG, Herold N (2018) Nucleobase and nucleoside analogues: resistance and re-sensitization at the level of pharmacokinetics, pharmacodynamics and metabolism. Cancers 10:240. https://doi.org/10.3390/cancers10070240
16. Thomson JM, Lamont IL (2019) Nucleoside analogues as antibacterial agents. Front Microbiol. https://doi.org/10.3389/fmicb.2019.00952
17. Doering AM, Jansen M, Cohen SS (1966) Polymer synthesis in killed bacteria: lethality of 2',3'-dideoxyadenosine. J Bacteriol 92:565–574. https://doi.org/10.1128/jb.92.3.565-574.1966
18. Beskid G, Eskin B, Cleeland R, Siebelist J, Cappetta AJ, Hill AD, Geiger RH (1981) Antibacterial activity of 2',3'-dideoxyadenosine in vitro and in vivo. Antimicrob Agents Chemother 19:424–428. https://doi.org/10.1128/AAC.19.3.424
19. Shin SJ, Collin MT (2008) Thiopurine drugs azathioprine and 6-mercaptopurine inhibit *Mycobacterium paratuberculosis* growth *in vitro*. Antimicrob Agents Chemother 52(2):418–426. https://doi.org/10.1128/AAC.00678-07
20. Liu F, Ma R, Riordan SM, Grimm MC, Liu L, Wang Y, Zhang L (2017) Azathioprine, mercaptopurine, and 5-aminosalicylic acid affect the growth of IBD-associated *Campylobacter* species and other enteric microbes. Front Microbiol. 8(527). https://doi.org/10.3389/fmicb.2017.00527
21. Sommerville L, Krynetsky EY, Krynetskaia NF, Beger RD, Zhang W, Marhefka CA, Evans WE, Kriwacki RW (2003) Structure and dynamics of thioguanine-modified duplex DNA*210. J Bio Chem 278(2):1005–1011. https://doi.org/10.1074/jbc.M204243200
22. Afifi SI (2010) Studies on Corynebacterium pseudotuberculosis, Staphylococcus aureus and Staphylococcus aureus subsp. Anaerobius isolated from sheep skin abscesses in Beni Suef Governorate. J Vet Med Res. 20(1):149–153. https://doi.org/10.21608/jvmr.2020.77592
23 Santiago LB, Alves FSF, Pinheiro RR, Santos VWS, Rodrigues AS, Chapaval L, Brito IF, Sousa FGC (2010) *In vitro* evaluation of the susceptibility of *Corynebacterium pseudotuberculosis* to different kinds of antisseptics and disinfectants. Arq Inst Biol. 7(4):593–600. https://doi.org/10.1590/1808-1657v77p5932010
24 Weinstein MP (2018) M07 - Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 11th edn. Clinical and Laboratory Standards Institute, Wayne, Pennsylvania
25. Hazan R, Que YA, Maura D, Rahme LG (2012) A method for high throughput determination of viable bacteria cell counts in 96-well plates. BMC Microbiol. 12(259). https://doi.org/10.1186/1471-2180-12-259
26. Sarker SD, Nahar L, Kumarasamy Y (2007) Microtire plate-based antibacterial assay incorporatinf resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods 42(4):321–324. https://doi.org/10.1016/j.ymeth.2007.01.006
27. Zander J, Besier S, Faetke S, Saum SH, Muller V, Wichelhaus TA (2010) Antimicrobial activities of trimethopin/sulfamethoxazole, 5-iodo-2'-deoxyuridine and rifampicin against *Staphylococcus aureus*. Int J Antimicrob Agents 36(6):562–565. https://doi.org/10.1016/j.ijantimicag.2010.08.007
28. Join-Lambert OF, Ouache M, Canioni D, Beretti JL, Blanche S, Berche P, Kayal S (2006) *Corynebacterium pseudotuberculosis* necrotizing lymphadenitis in a twelve-year-old patient. Pediatr Infect Dis J 25:848–851. https://doi.org/10.1097/01.inf.0000234071.93044.77