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New sequence type ST3756 of *Staphylococcus aureus* subspecies *anaerobius* as the causative agent of abscessing lymphadenitis in sheep

Sona Slosarkova[a], Jaroslav Bzdil[b], Katerina Nedbalcova[a], Jan Matiasovic[a,*], Petr Fleischer[a], Stanislav Stanek[c]

[a] Department of Immunology, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic
[b] Department of Special Microbiology, State Veterinary Institute Olomouc, Jakoubka ze Stribra 1, 779 00 Olomouc, Czech Republic
[c] Department of Technology and Breeding Technique of Farm Animals, Institute of Animal Science, Pratelstvi 815, 104 00 Prague, Czech Republic

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ABSTRACT

Morel's disease is a form of abscessing lymphadenitis of sheep and goats caused by *Staphylococcus aureus* subspecies *anaerobius*. In Europe and Africa, the disease is linked to *S. aureus* of multilocus sequence type 1464. In an outbreak recorded in 2015 in a flock of 530 animals in the district of Nymburk, Czech Republic, Europe, the causative agent was cultured and subsequently confirmed by Maldi–TOF. Neither antibiotic therapy nor surgical interventions met any success, although the strain isolated was found to be sensitive to antibiotics used. Vaccination and revaccination with inactivated autogenous vaccine administered subcutaneously was relatively successful. Subsequent multilocus sequence typing revealed the presence of new *S. aureus* sequence type 3756, different from 1464 in three out of seven genes typed. The isolate thus represents a new sequence type of *Staphylococcus aureus* ssp. *anaerobius* which should be considered as a causative agent of Morel's disease.

1. Introduction

Superficial abscessing lymphadenitis or caseous lymphadenitis (CLA) in small ruminants is commonly associated with *Corynebacterium pseudotuberculosis* and the disease is referred to as pseudotuberculosis [1]. However, diseases caused by other pyogenic microorganisms may also be present in the form of abscessing lymphadenitis. One of these microorganisms is *Staphylococcus aureus* and its subspecies, *S. aureus* ssp. *anaerobius,* which was identified as a causative agent of superficial abscessing lymphadenitis, known as Morel's disease [2]. It was also reported in human patients in South Australia [3]. Nowadays, this disease is described in sheep in African countries such as the Sudan, Ethiopia and Kenya, and also in Saudi Arabia. European countries have reported cases of this disease in Poland, Hungary, Croatia, Denmark and Spain [4–7]. Localization of abscesses is variable, but usually they are subcutaneous. Their content is viscous, yellowish and odorless [4].

S. aureus ssp. *anaerobius* strains can be identified in detail by Multilocus Sequence Typing (MLST). The sequence types may vary depending on the geographical or host origin of *S. aureus* ssp. *anaerobius* isolates. For example, Elbir et al. [5] published a diagram containing 22 different sequence types. Of these, 4 sequential types were isolated from sheep. In the Sudan, they defined a completely new sequence type (ST), ST1464, in 17 sheep isolates. De la Fuente et al. [8] examined 94 strains of *S. aureus* ssp. *anaerobius* isolated from abscesses of small ruminants in Spain, Denmark, Italy and the Sudan and detected in all cases the same sequence type ST1464. They conjectured that abscessing disease is caused by a single sequence type ST1464. However, this paper describes a case of rapid spreading Morel's disease which affected most sheep in a big flock in the Czech Republic and was caused by the new sequence type *S. aureus,* ssp. *anaerobius* ST3756.

2. Materials and methods

2.1. Animals

The affected flock consisted of 170 adult sheep (Romanov sheep, Romanov crossbred with Lacaune breed and purebred East Friesian sheep), 60 young sheep, approximately 300 lambs (in summer 2015) and 15 goats. All the animals were housed in pens in new wooden sheep shelters with deep straw bedding. Animals had free access to pasture and in winter were also fed with hay, haylage and 0.5–1.0 kg of a mixture of grounded barley, oats and lupine per animal and day. The commercial sheep farm was located in Nymburk district, Czech Republic.

* Corresponding author.
E-mail address: matiasovic@vri.cz (J. Matiasovic).

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2.2. Necropsy

A young sheep with symptoms of CLA was slaughtered and examined in the State Veterinary Institute (SVI) Olomouc. Whilst conducting thorough necropsy, endoparasites were screened for by a flotation technique and the abscess was bacteriologically examined.

2.3. Bacterial identification

Suppuration from the abscess was spread on blood agar plates, endo agar plates and on Sabouraud agar plates supplemented with chloramphenicol (0.05 $g.l^{-1}$ of medium) and incubated for 24 and 48 h at 37 °C. Microaerophilic cultivation was performed on blood agar plates and Edwards agar plates (all culture media were from Trios s. r. o., Prague, Czech Republic) in an anaerobic chamber (BioMérieux sa, Marcy l´Etoile, France) with atmosphere generated by a GENbox microaer (BioMérieux sa, Marcy l´Etoile, France) for 48 h at 37 °C. Anaerobic cultivation was performed in an anaerobic chamber with atmosphere generated by a GENbox anaer (BioMérieux sa, Marcy l´Etoile, France) for 48 h at 37 °C.

Colonies which were grown after the described incubation were subcultured under all three atmosphere conditions for 24 h. Catalase and oxidase tests and Gram staining were performed. Colonies from all three atmosphere conditions were identified by Maldi-TOF (Bruker Daltonik GmbH, Bremen, Germany).

2.4. Multilocus sequence Typing (MLST) of S. aureus ssp. anaerobius

The sheep isolate was checked for its sequence type according to MLST-SA database recommendation [9]. DNA for PCR typing was isolated by a method described by Chomczynski and Rymaszewski [10]. PCR products for *arcC*, *aroE*, *glp*F, *gmk*, *pta*, *tpi* and *yqiL* genes were purified by PCR purification kit (Qiagen) and sequenced in both orientations by a commercial company (SEQme, Dobříš, Czech Republic). Mismatches to known alleles in *pta*, *tpi* and *yqiL* were confirmed by an independent PCR and sequencing.

2.5. Phylogenetic analysis

Phylogenetic analysis between the newly described isolate and known ruminant isolates of *Staphylococcus aureus* was performed as described by de la Fuente et al. [8]. Concatenated sequences of all alleles were used to construct a neighbor-joining tree with 500 bootstrap replicates in the software package MEGA7 [11].

2.6. Antimicrobial susceptibility testing

Resistance to antimicrobials was assessed according to a standardized microdilution method of the Clinical and Laboratory Standards Institute (CLSI) [12,13]. The minimum inhibitory concentrations (MICs) were determined for penicillin, ampicillin, ceftiofur, erythromycin, clindamycin, gentamicin, vancomycin, trimethoprim/sulfamethoxazole, enrofloxacin, tetracycline, florfenicol, rifampicin, oxacillin and cefoxitin. Quality control of the kits was performed by including the reference strain of *Staphylococcus aureus* ATCC 29213. Due to the fact that interpretative criteria for *S. aureus* ssp. *anaerobius* originating from sheep are unknown, the susceptibility or resistance of the strain were evaluated according to criteria based on human-derived clinical breakpoints by CLSI [12,13] and breakpoints in human medicine by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [14] (Table 1). Determination of susceptibility or resistance to individual tested antimicrobials is valid only in human medicine in the case of human infection by this pathogen. The antimicrobial treatment of sheep should be carried out on the basis of evaluation of MIC values, pharmacokinetic and pharmacodynamic properties of antimicrobials, previous antimicrobial treatment in the flock and other available information.

2.7. Biochemical analysis of sheep blood

Blood was collected from the jugular vein of 6 clinically healthy adult sheep without visible abscesses. Serum samples were examined for total protein, albumin, urea, total bilirubin, aspartate aminotransferase (AST), nonesterified fatty acids (NEFA), beta hydroxybutyrate (BHB), Ca, inorganic P, Mg, Zn and Cu, and thyroxine (T_4).

2.8. ELISA detection of antibodies against C. pseudotuberculosis

Detection of antibodies against *C. pseudotuberculosis* in serum of 15 sheep (two of which had a visible abscess) was performed by ELISA-Kit for Detection of Caseous Lymphadenitis Adenosis (HYPHEN BioMed., Zac Neuville Université, Neuville sur Oise, France) in an accredited veterinary laboratory.

2.9. Vaccine and vaccination

An autogenous vaccine from the *S. aureus* ssp. *anaerobius* strain isolated at the SVI Olomouc (a newly isolated sequence type) from the affected flock was prepared (Dyntec s. r. o., Terezín, CZ) as a formaldehyde inactivated bacterin in the concentration of 2.56×10^8 of bacteria per ml supplemented with 15% of aluminium hydroxide, saponin and thiomersal. The vaccine dose for sheep/goats was set by the producer at 2 ml. The vaccine was subcutaneously administered to all animals during their pregnancy before the lambing season 2016, for the first time in the middle of December 2015. The second dose was administered after three weeks. Subsequently, animals were vaccinated/boostered every six months with the same dose. Before the lambing season 2017, the flock was divided into two parts, the first part was vaccinated before lambing, the second after it. In 2018, the whole flock was vaccinated before lambing. Use of autogenous veterinary vaccine is not subject to IRB approval.

3. Results

3.1. Case description

The first signs of the disease (nodules in a scrotum) were observed by the farmer in a newly purchased ram in the autumn of 2014. The ram came from a Lacaune flock in Vsetín district, Czech Republic. Whereas the animal was born there, the flock was imported from France in the past decade. Superficial nodules were observed in some of the imported animals and also some of their offspring, but the disease was not investigated further.

The farmer in the district of Nymburk then noticed an abscess on the head of one lamb in March 2015. Later, the presence of *Manheimia* sp. was found by an accredited diagnostic laboratory. The flock was treated with amoxicillin trihydrate in a dose of 15 mg kg of body weight (BW) (Clamoxyl L.A. inj.; Pfizer s.r.o., Prague, CZ). In May 2015, abscesses on the skin and subcutaneous lymph nodes on head, neck and thorax were found in 30 lambs. During summer 2015, nearly 90% of all lambs and approximately 40% of the adult sheep were affected. Abscesses were also found on the legs and around the udders.

Because pseudotuberculosis was suspected, pus samples were investigated by an accredited diagnostic laboratory SVI Prague in June and July 2015 (6 and 9 samples, respectively). Four of the first six samples were culture positive for *S. aureus* ssp. *anaerobius*, in two of them also *Klebsiella oxytoca* or / and *Streptococcus ovis* or / and *Trueperella pyogenes* were detected. In the remaining 2 samples, only *Trueperella pyogenes* was found. In the next 9 samples investigated for the presence of *C. pseudotuberculosis* or / and *S. aureus*, only *S. aureus* was found in two cases. All animals were treated again with amoxicillin at the end of June and, later, some animals also with ceftiofur

Table 1
Breakpoint table for *Staphylococcus aureus* based on MICs by CLSI (human-derived MIC breakpoints for veterinary pathogens) [12] and EUCAST [14] or CLSI [13] (breakpoints for humans).

	CLSI [12,13]			EUCAST [14]		
	MIC ($mg\,l^{-1}$)			MIC ($mg\,l^{-1}$)		
	S	I	R	S	I	R
Penicillin	≤0.125	–	≥0.25	≤0.125	–	≥0.25
Ampicillin	–	–	–	–	–	–
Ceftiofur	–	–	–	–	–	–
Erythromycin	≤0.5	1-4	≥8	≤1	2	≥4
Clindamycin*	≤0.5	1-2	≥4	≤0.25	0.5	≥1
Gentamicin	≤4	8	≥16	≤1	–	≥2
Vancomycin	≤2	4-8	≥16	≤2	–	≥4
Trimetoprim/sulphamethoxazole	≤2/38	–	≥4/76	≤2/38	4/76	≥8/152
Enrofloxacin	–	–	–	–	–	–
Tetracycline	≤4	8	≥16	≤1	2	≥4
Florfenicol	–	–	–	–	–	–
Rifampicin	≤1	2	≥4	≤0.06	0.125-0.5	≥1
Oxacillin**	≤2	–	≥4	≤2	–	≥4
Cefoxitin**	≤4	–	≥8	≤4	–	≥8

S = Susceptible.
I = Intermediate.
R = Resistant.
* CLSI document M100 28S.
** Breakpoints for oxacillin and cefoxitin in the table are valid for *S. aureus* and *S. lugdunensis*.

(6.6 $mg\,kg^{-1}$ BW; Naxcel 200 $mg\,ml^{-1}$ inj.; Pfizer Limited, Kent, GB). Visible abscesses were incised, evacuated and treated with local application of chlortetracycline spray (Pederipra spray 20 $mg\,ml^{-1}$; Hipra S.A., Girona, Spain). All therapies had limited success and, therefore, necropsy of an affected sheep was performed in the SVI Olomouc in September 2015.

A veterinary specialist in sheep diseases was called to conduct further examinations. During her visit at the beginning of December 2015, the body condition of the animals was found to be slightly less than desired. The specialist took blood samples for biochemical examination and for the detection of antibodies against *C. pseudotuberculosis*. She also took three samples from pussy lymph nodes of the two sheep with a visible abscess. Again, in all three samples *S. aureus* ssp. *anaerobius* was found (SVI Olomouc). Following these findings, the whole flock was subjected to a vaccination program. Since 2017, separation of positive animals and their gradual removal from the flock have been applied.

3.2. Necropsy

Necropsy findings in a sheep with signs of CLA: Body condition of the animal was slightly deteriorated. On the right side of its head, between eye and ear, an abscess of 3 cm in diameter with a capsule filled with greenish, creamy pus was found. The subcutaneous tissue of the abdomen and the inside of the thighs was edematous. The mucous membranes of the oral cavity, esophagus and trachea were unchanged. Lungs, pleura, pericardium, myocardium, spleen, liver, kidney, bladder, ovaries, uterus, mammary gland and joints were also without gross changes, as well as reticulum, rumen, omasum, abomasum and colon. Mesenteric lymph nodes and locally mucous membrane of the small intestine were edematous.

3.3. Bacterial identification – incl. MLST, phylogenetic analysis

Bacterial colonies from the abscess grown on blood agar plates under anaerobic and microaerophilic conditions were of 0.5–2.0 mm diameter or 0.1–0.5 mm under aerobic conditions and of pale color with partial beta-hemolysis. Colonies grown after subculture on blood agar plates under anaerobic, microaerophilic and aerobic conditions were catalase positive and oxidase negative. Under a microscope, Gram positive cocci in clusters were visible. Colonies from all three atmosphere conditions were identified by Maldi-TOF as *S. aureus* ssp. *anaerobius*. Sequences obtained unveiled the identity of known alleles in genes *arcC*-102, *aroE*-219, *glpF*-204 and *gmk*-122. Mismatches defined new alleles in *pta*-456 (one mismatch to *pta*-13), *tpi*-422 (two mismatches to *tpi*-177 or *tpi*-220) and *yqiL*-502 (one mismatch to *yqiL*-160). New alleles were deposited in MLST-SA database. The isolate was assigned the sequence type ST3756 and is stored in the Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Brno, Czech Republic as CAPM 6559 strain. Among the other ruminant *S. aureus* ssp. *anaerobius* isolates, ST3756 is closely related to ST1464, which is another strain linked to Morel's disease (Fig. 1).

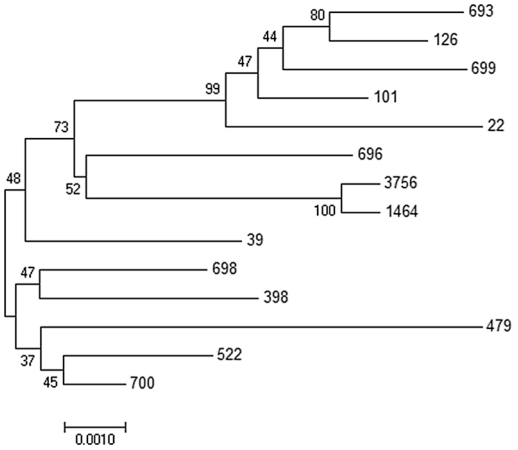

Fig. 1. Evolutionary relationships of ST3756 with other *S. aureus* sequence types.
The neighbor-joining consensus tree inferred from 500 bootstrap replicates is presented. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. Analysis reveals that ST3756 is the closest relative to ST1464, another strain linked to Morel's disease. Selection of sequence types for comparison was based on de la Fuente et al. [8].

Table 2
Results of antimicrobial susceptibility testing of strain *S. aureus* ssp. *anaerobius* based on human-derived interpretative criteria for veterinary pathogens (CLSI) or interpretative criteria for humans (EUCAST [14]) and results of QC strain *S. aureus* ATCC 29213 according to CLSI documents [12,13].

	S. aureus ssp. *anaerobius* MIC ($mg\,l^{-1}$)	Interpretation		Quality control according to CLSI [12,13] *S. aureus* ATCC 29213	
		CLSI [12,13]	EUCAST [14]	MIC ($mg\,l^{-1}$)	Acceptable QC ranges of MIC ($mg\,l^{-1}$)
Penicillin	≤0.06	S	S	0.5	0.25 – 2
Ampicillin	≤0.125	not rated	not rated	1	0.5 – 2
Ceftiofur***	1	not rated	not rated	0.25	0.25 – 1
Erythromycin	0.5	S	S	0.5	0.25 – 1
Clindamycin	≤0.125	S	S	0.25	0.06 – 0.25
Gentamicin	4	S	R	0.5	0.125 – 1
Vancomycin	2	S	S	0.5	0.5 – 2
Trimetoprim/sulphamethoxazole	0.5/9.5	S	S	0.5/9.5	≤0.5/9.5
Enrofloxacin***	0.25	not rated	not rated	0.06	0.03 – 0.125
Tetracycline	≤0.25	S	S	0.25	0.125 – 1
Florfenicol***	4	not rated	not rated	2	2 – 8
Rifampicin	0.03	S	S	0.008	0.004 – 0.015
Oxacillin*	0.125	S	S	0.25	0.12 – 0.5
Cefoxitin**	1	S	S	2	1 – 4

S = susceptible; R = resistant.
* *S. aureus* with oxacillin MIC values > $2\,mg.l^{-1}$ are mostly methicillin resistant due to the presence of the *mecA* or *mecC* gene.
** *S. aureus* with oxacillin MIC values > $4\,mg.l^{-1}$ are methicillin resistant, mostly due to the presence of the *mecA* or *mecC* gene.
*** Ceftiofur, enrofloxacin and florfenicol are veterinary specific antimicrobials – human derived criteria or criteria for human medicine are not defined.

3.4. Antimicrobial susceptibility

The results of antimicrobial susceptibility testing of the described strain *S. aureus* ssp. *anaerobius* are shown in Table 2. Table 2 contains the MICs values determined for individual antimicrobials for the strain *S. aureus* ssp. *anaerobius,* including interpretation of results by classification of the strain as susceptible or resistant to individual antimicrobials based on clinical breakpoints. The results of quality control testing with the reference strain *S. aureus* ATCC 29213 are also shown in Table 2. Although the breakpoints defined by CLSI [12,13] and EUCAST [14] are different for some antimicrobials, the categorization of the strain according to CLSI [12,13] and EUCAST [14] was different only for gentamicin. The strain *S. aureus* ssp. *anaerobius* is susceptible to all tested antimicrobials by CLSI [12,13] criteria but according to EUCAST [14] it is susceptible to tested antimicrobials with the exception of gentamicin to which the strain is resistant.

3.5. Biochemical examination

Results of almost all examined parameters showed no problems with the exceptions of the concentrations (x ± SD) of zinc ($8.6 \pm 1.0\,\mu mol.l^{-1}$) below the minimal reference values and urea ($3.6 \pm 0.7\,mmol.l^{-1}$) and albumin ($28.2 \pm 1.8\,g.l^{-1}$) just above the minimal reference values [15] and lower phosphorus concentrations in some animals ($1.9 \pm 0.3\,mmol.l^{-1}$).

3.6. Ruling out of C. pseudotuberculosis as a causative agent

From 15 examined serum samples eleven samples were diagnosed as serologically negative. Three samples were dubious and one was assigned as positive. However, bacteriological examination of cutaneous lesions on the animal tested positive by ELISA did not reveal any presence of *C. pseudotuberculosis*.

3.7. Effect of vaccination – according to the farmer

After the second dose of the vaccine, i.e. after primovaccination, the disease began to recede before the lambing season 2016. Offspring of the ewes which had been primovaccinated or revaccinated before parturition in 2017 developed hardly any clinical signs, most of lambs whose mothers have been vaccinated after parturition developed clinical signs, although they were vaccinated and revaccinated at the age of 4 and 7 weeks in the same way as the lambs of the first part of the flock. Abscesses were only observed in about 7% lambs in 2018, mostly classified as generally weaker animals.

4. Discussion

The symptoms of Morel's disease are very similar to those of pseudotuberculosis and are characterized by abscess formations in major superficial lymph nodes [4,5,16,17]. These similarities in the clinical picture, along with the fact that pseudotuberculosis is a better known and more common disease, frequently result in misdiagnosis of this disease. The incidence of Morel's disease in the Czech Republic has not yet been described. The main differences in the clinical picture were related to the bigger average number and size of abscesses per animal and faster infection spread in the flock [7,18]. Isolation and identification of the causative agent is still needed to confirm the diagnosis. The relatively rapid expansion of clinical signs was also evident from the description of the affected flock.

The borderline values of urea and albumin indicate short- and long-term problems in saturation of animals by nitrogenous substances, as well as subliminal concentrations of zinc and low phosphorus concentrations in some animals are the proof of inadequate saturation of animals by these elements. Lower body condition and deficiencies found in the area of nitrogenous substances and mineral nutrients possibly further facilitated a rapid spread of the infection among the animals [19]. Sufficient zinc and protein saturation are essential for the physiological function of the immune system. Their deficiency leads to a reduction in the level of both cellular and humoral immune responses [20,21].

However, for definitive differentiation between Morel's disease and pseudotuberculosis, it was necessary to identify the bacterium present in the affected tissues. Whilst the test on some animals was dubious, and one animal tested as positive for the antibody against *C. pseudotuberculosis*, this pathogen was not revealed during cultivation. On the other hand, in samples taken from diseased animals and from the abscess during necropsy, *S. aureus* (ssp. *anaerobius*) was repeatedly found to be present. The different findings of the 1 st and 2nd bacteriological examination were related to the fact that in the first samples the cause of the disease was still searched for, while the second examination was targeted specifically at the cause of the abscessing lymphadenitis, i.e. in particular to confirm or refute the presence of *C. pseudotuberculosis* or / and *S. aureus*. Other bacteria isolated from the first sampling, namely

Klebsiella oxytoca, *Streptococcus ovis* and *Trueperella pyogenes*, can be considered to be a secondary microflora. We thereby concluded that the flock was definitely affected by Morel's disease.

The isolated strain was found to be susceptible to penicillin, ampicillin, ceftiofur, erythromycin, clindamycin, vancomycin, trimethoprim/sulfamethoxazole, enrofloxacin, tetracycline, florfenicol, rifampicin, oxacillin and cefoxitin. In spite of this fact, the initial amoxicillin therapy given to the affected animals could have been effective, but it failed, probably because the antibiotic did not sufficiently penetrate into the abscesses [22]. Even ceftiofur was used with only limited success.

Importantly, vaccination with the autogenous vaccine was partially efficient. The key role of passive transfer of immunity through colostrum was shown by the fact that only ewes which had been boostered before parturition transferred immunity to their lambs based upon clinical signs. The vaccination against Morel's disease was previously successful when it was used in the Sudan [23].

During further identification of *S. aureus* ssp. *anaerobius* strain by multilocus sequence typing, new alleles were found in *pta*, *tpi* and *yqiL*, and the strain was assigned as the new sequence type ST3756. Phylogenetic analysis revealed its close relationship with another *S. aureus* ssp. *anaerobius* sequence type ST1464, known as the causative agent of Morel's disease in Europe and Africa [4,5]. Thus the ST1464 cannot be the sole sequence type causing this disease as previously conjectured by de la Fuente et al. [8].

Although the ST3756 was now identified for the first time in the Czech Republic, it is possible that this strain is already present in some infected sheep and goat flocks around Europe because of the origin of the initially infected ram and the ease of animal transfer within the EU. The presented case highlighted the predominant importance of health status checks and quarantine of newly purchased animals before they are introduced into an established flock.

Declarations of interest

None.

Acknowledgments

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