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Isolation of *Staphylococcus aureus* L Forms from Experimentally Induced Bovine Mastitis†

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Bacterial L forms were isolated from milk samples of dairy cattle infected experimentally with *Staphylococcus aureus*. Initially, bacterial L forms were induced in vitro from 12 of 44 *S. aureus* strains isolated from bovine mastitis. Cows were experimentally infected in two experiments with strains shown in vitro to be easily inducible to L form and with *S. aureus* Newbould 305. Each quarter of the mammary gland was infected with either 10^3 or 10^6 CFU of the test strains. Treatment was initiated with 100,000 U of penicillin G per quarter at the first signs of clinical mastitis. Milk samples were collected daily and cultured on bovine blood agar and PPLO agar (Difco Laboratories, Detroit, Mich.) with 10% horse serum and 5% NaCl. Staphylococcal L forms were isolated from milk samples collected from infected glands in both experiments after antibiotic therapy. Glands with the highest concentrations of leukocytes and bacteria were most likely to yield L forms in milk samples after treatment was initiated. Cows harboring L forms typically yielded parental organisms after cessation of antibiotic therapy. No detectable changes occurred in antibiotic susceptibilities, coagulase production, or biochemical activities in strains induced to L form followed by reversion to the parental form. These results demonstrated that L forms can occur during treatment of bovine mastitis and that L forms may be one explanation for the poor response of staphylococcal bovine mastitis to antibiotic therapy.

Bovine mastitis is a disease of major economic importance to the dairy industry. *Staphylococcus aureus* is a leading cause of mastitis and one of the most difficult udder pathogens to control. Resistance to antibiotics is a factor in treatment failures. However, poor response to treatment is often encountered when in vitro susceptibility tests indicate that the organism is susceptible to the drug of choice.

A variety of explanations have been put forth to explain therapy failures. Clinical staphylococcal mastitis typically results in marked inflammation, leading to damage of epithelial tissues and scar tissue formation (1, 7). Microorganisms sequestered within these areas are physically protected from contact with antibiotics (1, 5). In addition, Craven and Anderson (5) have shown that organisms in these areas are often metabolically inert and are not susceptible to β-lactam antibiotics. *S. aureus* is capable of intracellular survival within the phagocytes protected from other host defenses and antibiotics (5).

Under certain conditions, antibiotic therapy may result in formation of bacterial L forms. L forms have been implicated in a variety of diseases, including chronic urinary tract infections (4, 14), rheumatic fever (8), septicemia (6, 10), osteomyelitis (12), and tuberculosis (12). However, conclusive evidence of their role in these diseases has not been demonstrated. The most convincing evidence of a role for L forms in disease indicates that they may serve as a transition state for the organism, allowing it to escape therapy and reemerge in parental form as more favorable conditions return. Studies on L forms of *Mycobacterium tuberculosis* indicate a role for reverted L forms in remanifestation of chronic infections (12). Evidence of *S. aureus* L forms in persisting cases of osteomyelitis indicates a possible role for reversion of L forms to parental forms (12). L forms of *Nocardia caviae* have been reported to contribute to the virulence of that organism without the necessity of reversion to the parental form (3).

During the course of untreated bovine mastitis, inflammation and damage to secretory tissue result in increases in electrolytes, pH, and proteins in serum. Treatment with β-lactam antibiotics under these conditions may allow development of L forms. This study was undertaken to determine whether L forms of *S. aureus* occur during treatment of clinical mastitis.

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MATERIALS AND METHODS

Bacteria and media. *S. aureus* strains were from our Mastitis Research Laboratory culture collection and were originally isolated from cases of bovine mastitis. Organisms were maintained frozen at −20°C and cultured from stocks on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% bovine blood (TBA) at 35°C. PPLO broth (Difco Laboratories, Detroit, Mich.), supplemented with 0.5% yeast extract (Difco), 10% horse serum (Sigma Chemical Co., St. Louis, Mo.), and 5% NaCl with or without agar, was used for cultivation of L forms. All strains tested were susceptible to penicillin by agar disk diffusion testing.

L form induction. Forty-four *S. aureus* strains were tested for induction to L forms. Organisms were suspended in Trypticase soy broth to a turbidity approximating a 0.5 McFarland standard and incubated for 2 h to assure that cells were in log phase. To induce L forms, 0.1 ml of the Trypticase soy broth suspension was added to 5 ml of PPLO broth supplemented with 10% horse serum and 5% NaCl containing 100 U of procaine penicillin G (Sigma) per ml. Samples were taken after 10 min of incubation and again after 24 h; 0.1 ml of sample was plated to supplemented PPLO agar. Plates were incubated at 35°C and observed

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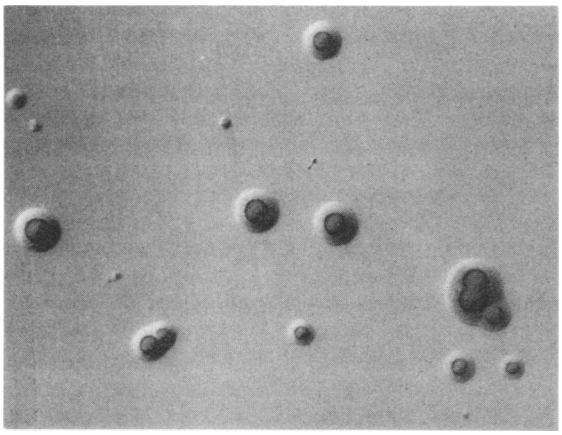

FIG. 1. Typical L-form colonies of *S. aureus* on PPLO agar. Magnification, ×25.

daily for 7 days for typical fried egg L-form colonies. Two strains (A and B) shown to be easily inducible to L form by the above procedure were used for further testing.

Reversion of L forms. L-form colonies were subcultured to supplemented PPLO broth and incubated for 48 h at 35°C. Subcultures were made from the PPLO broth to PPLO agar and TBA. Repeated subculture of L-form colonies resulted in reversion to the parental form after three to five subcultures. Reverted strains of A and B were tested for coagulase, hemolytic patterns, and antibiotic susceptibility patterns by standard methods and compared with parental strains.

In vivo induction of L forms. Two separate experiments were conducted to determine whether L forms were induced during treatment of clinical mastitis. In the first, four cows were experimentally infected: two with strain A and two with strain B. Organisms were grown on TBA for 24 h, harvested in physiologic saline, and washed twice before infusion. Cows were infused in each quarter of the mammary gland with 10^6 CFU of the test organisms via the teat end. Cows with no recent history of *S. aureus* infection were chosen for challenge and were bacteriologically negative in all quarters before infusion. Treatment was initiated at first signs of clinical mastitis. Cows were treated twice daily for 3 days in each gland with a lactating-cow product containing 100,000 U of penicillin G (Aqua-mast; Kendall, Agricultural Products Division, Boston, Mass.). Milk samples were taken just before initial treatment and at each subsequent milking. Cows were sampled twice daily for 15 days. Samples were plated within 1 h to supplemented PPLO agar (0.1 ml) and TBA (0.01 ml). PPLO plates were incubated at 35°C and checked daily for L-form colonies. Representative colonies were reverted as described above to ensure that they originated from parental strains. TBA plates were also incubated at 35°C for 48 h and examined for parental *S. aureus* colonies.

In the second experiment, six cows were infected with approximately 10^3 CFU: two with strain A, two with strain B, and two with *S. aureus* Newbould 305. Quarter milk sample collection, treatment, and culture were as described above. In addition, quantitation of bacterial numbers and somatic cell counts were performed. Somatic cell counts were determined with an electronic somatic cell counter (Fossomatic Milk Analyses; A. S. Foss, Inc., Hillerød, Denmark) as described by Madsen (9). Bacterial numbers were determined by standard plate count.

Determination of antibiotic concentration. A disk plate assay was used to determine the concentration of penicillin in each quarter sample after treatment was initiated. Samples were taken just before the next scheduled dose, and a sample was frozen for assay. All samples were assayed together by following the procedures outlined in the *Manual of Clinical Microbiology* (2). Briefly, 150-mm (diameter) petri plates were filled to a level of approximately 4 mm with Mueller-Hinton II (BBL) agar, plates were swabbed uniformly with *S. aureus* ATCC 25923, and six blank paper disks were placed on each plate. Standard concentrations of penicillin, from 0.1 to 1,000 U/ml, were added to the blank disks at a volume of 10 μl per disk. A standard curve was developed on the basis of zone diameters resulting from the known antibiotic concentrations. Similar plates were prepared in duplicate for milk samples, and 10-μl samples were added to blank disks. Zone diameters were measured, and antibiotic concentrations were determined from the standard curve. Quarter sample concentrations were expressed as the mean concentration of antibiotic per quarter at each sample time.

RESULTS

Of 44 *S. aureus* strains tested in vitro, 12 were induced to L form under the conditions described. Typical L-form colonies are shown in Fig. 1. L forms were isolated from PPLO broth containing 100 U of penicillin per ml after both 10 min and 24 h of exposure to penicillin. Table 1 shows relative numbers of L forms isolated from the 12 positive strains. *S. aureus* Newbould 305, a strain widely used in mastitis research, was induced to L form. However, L-form colonies of this strain were unstable and reverted to parental forms within 24 to 48 h after first isolation. L forms from other strains were stable to passage on PPLO agar for at least three to four subcultures. Two strains, no. 2 and 7 (Table 1), were chosen for further experiments and designated strains A and B, respectively. Reversion of freshly isolated L forms was best accomplished by subculturing to PPLO broth for 1 to 5 days, with subculturing to supplemented PPLO agar and TBA daily. Reverted strains of A and B retained the original coagulase, hemolytic, and antibiotic susceptibility patterns.

Results of the first in vivo experiment are summarized in

TABLE 1. Approximate numbers of L-form colonies isolated from strains induced to L form by exposure to 100 U of penicillin per ml

MRL no.[a]	Strain	No. of L-form colonies at[b]:	
		10 min	24 h
67	1	0	1+
13	2[c]	3+	3+
20	3	1+	0
39	4	2+	0
66	5	1+	0
12	6	0	1+
118	7[c]	2+	2+
305	8	0	2+[d]
112	9	2+	2+
18	10	0	1+
62	11	2+	2+
41	12	1+	1+

[a] Mastitis Research Laboratory culture number.
[b] Scale: 1+, 1 to 10 CFU/0.1 ml of sample; 2+, 10 to 50 CFU/0.1 ml of sample; 3+, 50 to 100 CFU/0.1 ml of sample; 4+, >100 CFU/0.1 ml of sample.
[c] Strain chosen for cow experiments.
[d] L-form colonies reverted quickly to parental forms.

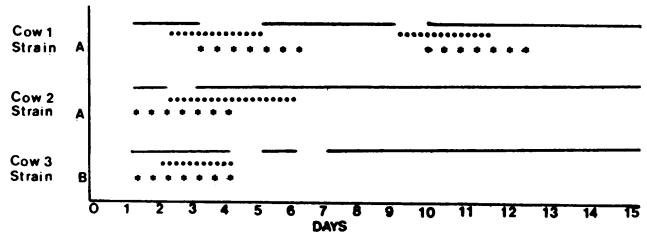

FIG. 2. Isolation of L forms from cows experimentally infected with 10^6 CFU of *S. aureus* per quarter and treated with penicillin. Cows were inoculated on day zero. Treatment was initiated with 100,000 U of penicillin per quarter every 12 h on the day indicated. ———, Parental forms isolated; · · · ·, L forms isolated; ***, treatment days.

Fig. 2. Four cows were experimentally infected with strains A and B. All four cows had clinical mastitis in each quarter within 24 h of infusion. One of the cows receiving strain B developed gangrenous mastitis and died 2 days after infusion. L forms were isolated from the remaining three cows within 24 h of initiation of therapy. Parental forms of *S. aureus* were not detectable in some samples from cows 1 and 2 infected with strain A. However, L forms were detectable in these samples. No organisms were detected in cow 3 (strain B) in samples from day 4. However, this cow did shed parental forms 24 h after treatment ended. Interestingly, parental forms of *S. aureus*, as well as L forms, were present during treatment despite presumed high levels of antibiotics in the gland. Cow 1 (strain A) was retreated on day 8. L forms were the only organisms detected on samples from day 9. Parental forms reemerged on day 10 despite continued treatment. Representative L-form colonies of strains A and B were reverted as described above. Reverted colonies retained the original coagulase, hemolytic, and antibiotic susceptibility patterns.

Results from the initial in vivo experiment prompted a more detailed study. Six lactating cows were infected as described above. Highest cell counts were detected in cows infected with strain A (Table 2). Cell counts were elevated in all cows within 12 to 24 h of infection and ranged from 1×10^6 to 1.1×10^7 CFU/ml just before initiation of treatment. Reduction in cell count was gradual after initiation of therapy and remained highest in cows infected with strain A. CFUs of *S. aureus* per milliliter present just before, during, and after treatment of the three groups are shown in Tables 3, 4, and 5. CFUs of L forms per milliliter are also listed in these tables. One cow infected with *S. aureus* Newbould 305 (cow 2) appeared to clear the infection spontaneously before treatment (Table 5). Numbers of *S. aureus* dropped below detectable levels in all but one quarter by the second milking after infusion of organisms. No L forms were isolated from this animal. Occurrence of L forms was more erratic in this experiment than in the first in vivo experiment represented in Fig. 2. The highest numbers of L forms were found just after initiation of therapy and were often accompanied by parental forms, as well as intermediate or damaged forms that could grow, albeit poorly, on TBA. The intermediate forms appeared as small colonies on PPLO agar similar in size initially to L forms. They had the grainy or lacy appearance characteristic of the outside edge of typical L-form colonies but lacked the dense center. These intermediate forms did not adhere to the agar as did L-form colonies and reverted to typical parental forms on subculturing to either blood agar or PPLO agar. Figure 3 shows a mixture of typical L forms with intermediate forms on PPLO agar. Cows in the first experiment were given a much higher infecting dose of *S. aureus*, and this may have contributed to the higher prevalence of L forms isolated. L forms were more prevalent in cows with the highest cell counts and CFU of parental forms before treatment. Antibiotic levels in the milk samples are shown in Table 6. Levels fluctuated from milking to milking. Samples were taken just before the next scheduled treatment and represent the lowest levels present during the treatment period.

Antibiotic levels were lowest in cows with lower cell counts (Table 6). The lowest levels were recorded for cow 2, infected with *S. aureus* Newbould 305. This cow had low cell counts and cleared the infection rapidly. Only two cows of the six in the second in vivo experiment remained infected at the end of the sampling period, and retrieval of organisms from these animals was sporadic. Both of these cows were infected with strain A, and both had L forms present in milk samples at various times during the experiment. Cows

TABLE 2. Mean somatic cell count from cows infected with 10^3 CFU of the indicated organism

Postinoculation milking no.	Somatic cell count/ml (10^3)[a]:					
	Strain A		Strain B		*S. aureus* Newbould 305	
	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2
0[b]	167	389	385	809	733	161
1	11,065	3,469	465	753	8,665	550
2	**11,785**	6,427	1,609	4,181	8,923	1,117
3	**11,017**	**8,286**	1,943	8,677	**6,955**	1,046
4	**5,019**	**2,180**	3,703	2,934	**3,437**	**410**
5	**9,026**	**3,737**	10,495	4,898	**5,057**	**983**
6	**1,537**	**1,154**	**4,622**	**4,715**	**2,065**	**132**
7	4,001	**2,227**	**6,812**	**4,463**	**2,472**	**376**
8	2,331	1,128	**3,059**	**2,921**	2,316	**180**
9	5,442	1,383	**3,111**	**2,723**	2,015	618
10	2,552	3,005	**2,295**	**2,868**	1,593	165
11	3,112	1,900	1,991	989	1,145	328
12	1,808	993	891	715	813	108
13	1,250	729	666	355	897	79
14	2,329	732	688	461	813	63

[a] Boldfaced numbers indicate treatment days.
[b] Mean somatic cell count before infection.

TABLE 3. CFU per milliliter from TBA and PPLO agar[a] by quarter from cows infected with 10^3 CFU of strain A per quarter

Cow, quarter, and medium[b]	CFU/ml at postinoculation milking no.:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cow 1														
RF-TBA	1×10^6[c]	1×10^3	4×10^2	1×10^2	0	0	0	0	3×10^2	0	0	0	0	7×10^2
RF-PPLO	—[d]	5×10^3	1×10^2	0	0	5×10^1	0	0	3×10^1	0	0	9×10^1	0	0
LF-TBA	1×10^6	7×10^2	4×10^2	1×10^2	0	0	0	0	0	0	0	0	0	1×10^3
LF-PPLO	—	4×10^2	0	0	0	0	0	0	2×10^1	0	0	0	0	0
LR-TBA	2×10^6	0	1×10^2	0	0	0	0	0	0	0	0	0	0	8×10^2
LR-PPLO	—	5×10^3	1×10^2	0	0	5×10^2	0	0	0	0	0	0	2×10^1	0
RR-TBA	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RR-PPLO	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cow 2														
RF-TBA	1×10^5	1×10^4[c]	0	0	0	0	0	0	0	0	0	0	0	0
RF-PPLO	—	—	0	0	0	0	0	0	0	0	0	0	0	0
LF-TBA	1×10^5	1×10^4	0	0	0	0	0	0	0	0	0	0	0	0
LF-PPLO	—	—	0	0	0	0	0	0	0	0	0	0	0	0
LR-TBA	2×10^5	1×10^5	2×10^3	3×10^2	0	0	0	0	0	3×10^2	0	4×10^2	8×10^1	1×10^4
LR-PPLO	—	—	0	0	0	5×10^1	0	0	2×10^1	0	0	0	0	0
RR-TBA	1×10^5	1×10^5	7×10^2	5×10^2	0	1×10^2	0	0	0	0	0	0	0	0
RR-PPLO	—	—	5×10^1	0	1×10^1	0	0	3×10^1	0	0	0	0	0	0

[a] CFU from PPLO agar indicate L forms only.
[b] Quarter designations: RF, right front; LF, left front; LR, left rear; RR, right rear.
[c] Milking when therapy was initiated.
[d] —, Sample not taken.

TABLE 4. CFU per milliliter from TBA and PPLO agar[a] by quarter from cows infected with 10^3 CFU of strain B per quarter

Cow, quarter, and medium[b]	CFU/ml at postinoculation milking no.[c]:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cow 1														
RF-TBA	—	4×10^3	3×10^4	5×10^5[d]	2×10^5	2×10^4	0	2×10^3	3×10^2	0	0	0	7×10^1	0
RF-PPLO	—	—	—	—	—	2×10^3	7×10^2	8×10^2	0	0	0	5×10^1	0	0
LF-TBA	—	2×10^3	1×10^4	1×10^3	2×10^3	3×10^2	1×10^2	0	0	0	0	0	0	0
LF-PPLO	—	—	—	—	—	0	0	0	0	0	0	0	0	0
LR-TBA	—	2×10^4	2×10^3	4×10^3	3×10^4	4×10^2	1×10^2	0	0	0	0	0	0	0
LR-PPLO	—	—	—	—	—	3×10^1	0	0	0	0	0	0	0	0
RR-TBA	—	4×10^3	2×10^4	4×10^3	1×10^3	0	0	0	0	0	0	0	0	0
RR-PPLO	—	—	—	—	—	0	0	0	0	0	0	0	0	0
Cow 2														
RF-TBA	—	1×10^4	9×10^3	4×10^2[d]	3×10^2	0	0	0	0	0	0	0	0	0
RF-PPLO	—	—	—	—	—	0	0	0	0	0	0	0	0	0
LF-TBA	B													
LF-PPLO	B													
LR-TBA	—	1×10^3	5×10^1	3×10^4	1×10^3	0	0	0	0	0	0	0	0	0
LR-PPLO	—	—	—	—	—	0	0	0	0	0	0	0	0	0
RR-TBA	—	0	2×10^1	5×10^5	5×10^4	9×10^3	5×10^2	2×10^2	2×10^2	0	0	0	0	0
RR-PPLO	—	—	—	—	—	1×10^2	0	5×10^1	0	0	0	0	0	0

[a] CFU from PPLO agar indicate L forms only.
[b] Quarter designations: RF, right front; LF, left front; LR, left rear; RR, right rear.
[c] B, Blind quarter; —, sample not taken.
[d] Milking when therapy was initiated.

TABLE 5. CFU per milliliter from TBA and PPLO agar[a] by quarter from cows infected with 10^3 CFU of *S. aureus* Newbould 305 per quarter

Cow, quarter and medium[b]	CFU/ml at postinoculation milking no.:													
	1[c]	2	3	4	5	6	7	8	9	10	11	12	13	14
Cow 1														
RF-TBA	8×10^3	1×10^3	0	0	0	0	0	0	0	0	0	0	0	0
RF-PPLO	—[d]	0	0	0	7×10^2[e]	0	0	0	0	0	0	0	0	0
LF-TBA	1×10^4	1×10^3	0	0	0	0	0	0	0	0	0	0	0	0
LF-PPLO	—	—	0	0	0	0	0	0	0	0	0	0	0	0
LR-TBA	2×10^3	2×10^4	0	0	0	0	0	0	0	0	0	0	0	0
LR-PPLO	—	2×10^2	7×10^2	0	0	0	0	0	0	0	0	0	0	0
RR-TBA	1×10^3	0	0	0	0	0	0	0	0	0	0	0	0	0
RR-PPLO	—	0	0	0	0	0	0	0	0	0	0	0	0	0
Cow 2														
RF-TBA	1×10^3	0	0	0	0	0	0	0	0	0	0	0	0	0
RF-PPLO	—	—	0	0	0	0	0	0	0	0	0	0	0	0
LF-TBA														
LF-PPLO														
LR-TBA	3×10^3	0	0	0	0	0	0	0	0	0	0	0	0	0
LR-PPLO	—	—	0	0	0	0	0	0	0	0	0	0	0	0
RR-TBA	5×10^2	1×10^1	0	0	0	0	0	0	0	0	0	0	0	0
RR-PPLO	—	—	0	0	0	0	0	0	0	0	0	0	0	0

[a] CFU from PPLO agar indicate L forms only.
[b] Quarter designations: RF, right front; LF, left front; LR, left rear, RR, right rear.
[c] Milking when therapy was initiated.
[d] —, Sample not taken.
[e] Mixed L and parental forms were isolated from this sample.

infected with strain B and *S. aureus* Newbould 305 were bacteriologically negative for both L forms and parental forms at the end of the sampling period.

DISCUSSION

This study demonstrated that L forms do occur during treatment of experimentally induced *S. aureus* bovine mastitis and can be isolated from milk samples yielding negative results by traditional methods. L forms may serve as an intermediate stage, allowing the mastitis pathogen to withstand antibiotic therapy, persist in the mammary gland, and reemerge with remanifestation of the disease when therapy has ended. The low level of complement (13) and the impaired activity of phagocytes (11) within the gland may contribute to the ability of L forms to persist during infection. This explanation would add L-form induction to the list of possible explanations for the poor response of *S. aureus* mastitis to therapy. Many antibiotics available for treatment of bovine mastitis are cell wall active and capable of inducing L forms in a variety of microorganisms. The choice of antibiotic, particularly for acute *S. aureus* mastitis, may need to reflect the possibility of L-form induction. The variability seen between strains for induction to L forms suggests that herds harboring a large number of strains

TABLE 6. Mean penicillin concentration per quarter

Strain and cow	Mean penicillin concn/quarter (U/ml) at postinoculation milking no.:													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Strain A														
Cow 1	—[a,b]	46	95	55	500	50	360[c]	7.0	0	0	0	0	—	—
Cow 2	—	—[b]	90	35	200	43	95[c]	40	8.5	0	0	0	—	—
Strain B														
Cow 1	—	—	—	—	—[b]	10	80	15	95[c]	9.5	0	0	—	—
Cow 2	—	—	—	—	—[b]	45	95	55	90[c]	20	5	0	—	—
S. aureus Newbould 305														
Cow 1	—[b]	90	45	90	55	95[c]	46	9.5	0	0	—	—	—	—
Cow 2	—	—[b]	0	9.3	7.0	9.5[c]	0	0	0	0	—	—	—	—

[a] —, No sample taken.
[b] Initiation of treatment.
[c] Last treatment.

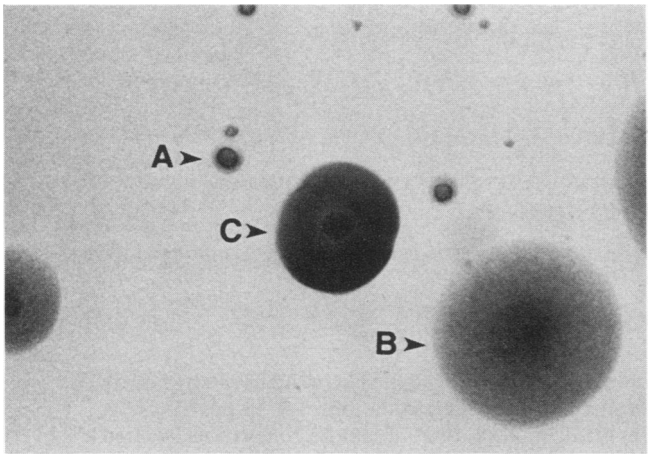

FIG. 3. Mixed culture of typical L-form colonies (A), intermediate colonies (B), and a partially reverted L-form colony (C) resembling the parent. Magnification, ×25.

easily inducible to L form could be at a higher risk for therapy failure and chronic infection. The higher incidence of L forms in cows infected with a large inoculum suggests that increased inflammation with increases in pH, electrolytes, and protein in serum may favor L-form induction in vivo. Further work is needed to determine the precise conditions necessary in the gland for L-form induction and their importance in the control of mastitis in dairy cattle.

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