

In Vitro and In Vivo Study of Stone Formation by *Corynebacterium* Group D2 (*Corynebacterium urealyticum*)

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***Corynebacterium* group D2 inoculated into normal human urine formed struvite crystals and an increase in pH and ammonium concentration after 24 h of incubation. Zinc disks dipped into a broth culture of this microorganism and inserted into the bladders of rats produced stones with a mean weight of 12.5 mg (ranging from 1 to 57.7 mg) after 12 days. Analysis of the infrared spectrum determined the stones to be composed of struvite. From these results it seems that stone formation by *Corynebacterium* group D2 may be possible both in vitro and in vivo, which may confirm a previous report involving these bacteria in human clinical encrusted cystitis.**

Phosphate stones, particularly struvite (ammonium magnesium phosphate: NH_4MgPO_4), are thought to develop in urinary tracts infected by urea-splitting bacteria such as *Proteus* species. The bacterial urease hydrolyzes urea, leading to hyperammonuria and the alkalization of urine, which causes supersaturation with struvite and calcium phosphate with consequent crystallization of struvite and apatite. This type of urinary stone has been shown to occur in vitro (2, 9) in human urine and in vivo (1, 8, 10) in the bladder of rats by using a highly urease-producing bacterial strain such as *Proteus mirabilis*. Recently we reported that *Corynebacterium* group D2, a gram-positive bacillus with a strong urease activity (3) and high resistance to most antimicrobial agents (5), was involved in cases of alkaline-encrusted cystitis (6), a chronic inflammatory condition of the bladder with deposits of ammonium magnesium phosphate on the surface and on the walls of a previously damaged bladder. Bacteriuria by *Corynebacterium* group D2 has been detected in up to 0.2% of the urine samples microbiologically studied in our laboratory. Although these bacteria do not always produce either encrusted cystitis or clinical disease, up to 60% of the isolations have clinical significance in our patients, most of whom suffer some kind of urological disturbance (J. M. Aguado, C. Ponte, R. Fernández Roblas, and F. Soriano, Abstr. Second Eur. Congr. Clin. Microbiol. 1985, 20/2). In this report, we show that urinary stones are formed in human urine inoculated with *Corynebacterium* group D2 as well as in a bladder model in rats infected by this microorganism.

MATERIALS AND METHODS

Bacterial strain. *Corynebacterium* group D2, García strain (ATCC 43044), isolated from urine in a patient with alkaline-encrusted cystitis was used throughout. *Escherichia coli* ATCC 25922 and *Proteus vulgaris* ATCC 6380 were also used.

Formation of urinary crystals (in vitro model). A fresh urine sample was obtained from one of us, who had no history of urinary stones or urogenital infection, and sterilized by Seitz filtration. Bacteria were grown aerobically at 37°C for 24 h in Mueller-Hinton broth; in the case of *Corynebacterium* group D2, the broth was enriched with 1%

Tween 80 and 10% sterile rabbit serum. One milliliter of a 1:100 dilution in human urine of an overnight culture of *Corynebacterium* group D2 was inoculated into 9 ml of the sterile urine. *E. coli* and *P. vulgaris* were inoculated in the same fashion but with a 1:1,000 dilution of the overnight culture. All inocula gave a final concentration of ca. 10^5 CFU/ml. A control of 10 ml of urine without bacteria was also studied. All tubes were incubated aerobically at 37°C for 24 h.

Ammonium (volumetric method), colony count, and pH were determined at the beginning of the experiment and after 7 and 24 h of incubation. Sediment was examined from samples at the same intervals, and crystals, if any, were identified both microscopically and with an infrared spectrometer (Perkin Elmer 457) by using a potassium bromide tablet.

Formation of stones in the bladder (in vivo model). An experimental urinary tract infection was established in a manner similar to that described previously by Vermeulen and Goetz (10). Female Wistar strain rats 180 to 200 g in body weight were used in all experiments. Rats were anesthetized with ether and the abdomen hair was shaved, and then the abdominal wall was thoroughly swabbed with povidone iodine. A small suprapubic incision was made at the abdominal wall, and the bladder was exposed and opened, followed by the immediate insertion of a zinc disk (6 mm in diameter) dipped into a 24-h broth culture of *Corynebacterium* group D2 or *E. coli*. Disks handled in this fashion carried ca. 10^5 organisms. A silk suture was used to close the incision of the bladder, peritoneum, muscle, and skin. Rats were maintained on water and chow ad libitum and sacrificed after 12 days to observe the development of the bladder stone. The mucous surface of the bladder, zinc disks, and stones, if any, were cultured aerobically on blood agar, and the gross appearance of the bladder was noted. Disks were dried overnight at 42°C, and the amount of stone formation was determined by subtracting the original weight of the disk from the weight of a disk plus its associated calculus. Where secondary stones were found in addition to the principal one on the zinc disk, these daughter stones were not included in the weights given in the results section. Stones were analyzed by infrared spectrometry.

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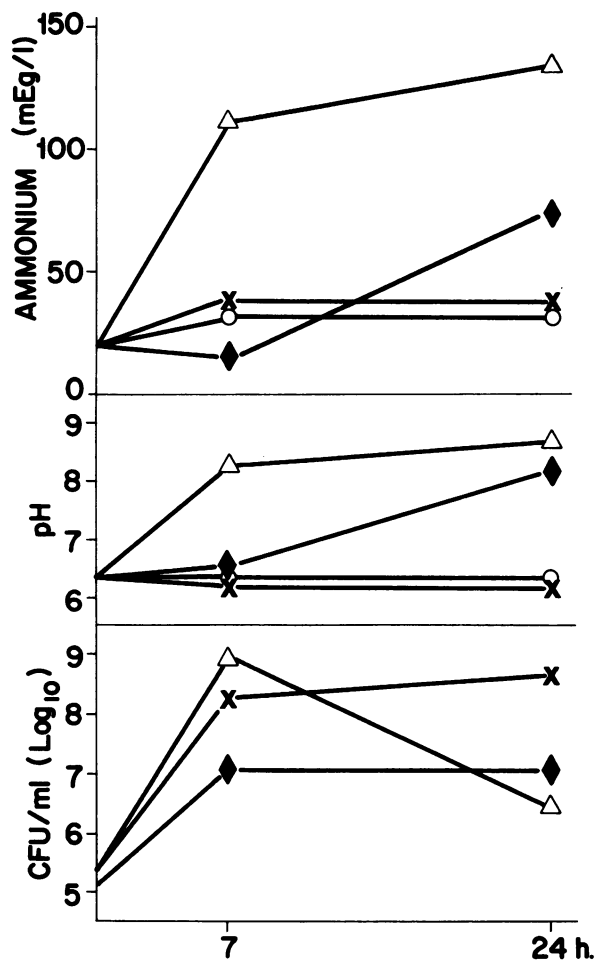


FIG. 1. Effect of bacterial growth in human urine. Ammonium concentration, pH, and colony count at 0, 7, and 24 h of incubation in presence of (○) no bacteria, (◆) *Corynebacterium* group D2, (×) *E. coli*, or (Δ) *P. vulgaris*.

RESULTS

Formation of urinary crystals in human urine (in vitro model). Figure 1 shows the results of a single experiment indicating the ammonium concentration, pH, and colony count in urines inoculated with *Corynebacterium* group D2, *P. vulgaris*, and *E. coli*. After 7 h of incubation, *P. vulgaris*

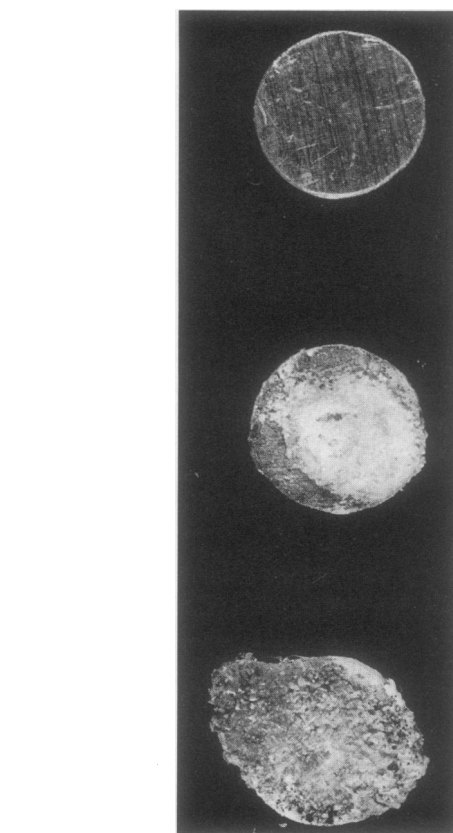


FIG. 2. Examples of bladder stones that formed on surgically inserted zinc disks that had been dipped into a broth culture of *Corynebacterium* group D2. Top, Zinc disk; middle, 19.9-mg stone; bottom, 38.6-mg stone.

increased the ammonium concentration, pH, and number of viable cells, and struvite crystals appeared which persisted after 24 h. *Corynebacterium* group D2 increased the ammonium concentration and pH after 24 h; struvite crystals appeared only after this period of time. On the other hand, in tubes containing *E. coli* or no bacteria there was no variation of the ammonium concentration and pH, and no crystals appeared after 24 h of incubation.

Formation of stones in the bladder (in vivo model). Table 1 shows the results obtained with the study of 34 animals inoculated in the bladder with zinc disks dipped in the bacterial cultures. In the first group, composed of 24 rats

TABLE 1. Results of the in vivo model of bladder infections in rats

Group (no. of animals)	Infected with:	Final culture (zinc disk and bladder)	No. of rats	No. with stones	Stone wt (mg)	
					Range	Mean
I (24)	<i>Corynebacterium</i> group D2	<i>Corynebacterium</i> group D2	14	14	1-57.7	12.5
		<i>Corynebacterium</i> group D2 plus other microorganism ^a	2	2	10.2-12.1	11.1
		<i>Streptococcus</i> group D (urease negative)	2	0		
		<i>Proteus</i> sp.	1	1	70.8	70.8
		Negative	5	0		
II (10) ^b	<i>E. coli</i>	<i>E. coli</i>	7	0		
		<i>E. coli</i> plus <i>Proteus</i> sp.	1	1	18.0	18.0

^a One *S. aureus* (urease positive) and one *Streptococcus* group D (urease negative) strain.

^b Two animals in this group died.

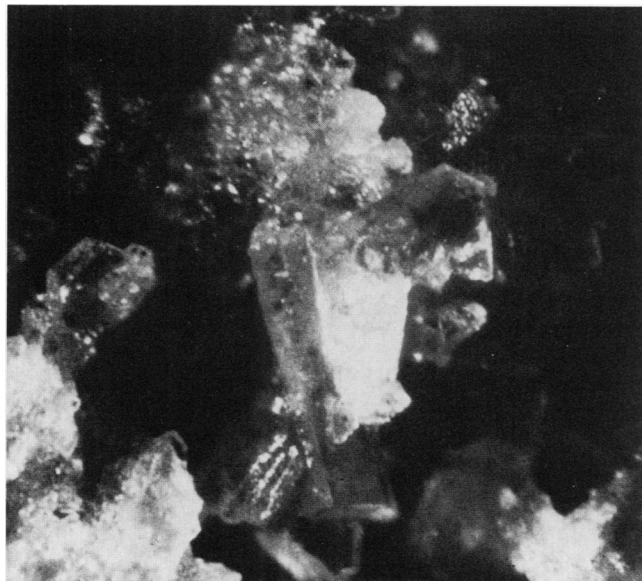


FIG. 3. Gross appearance of hard, gritty material induced by *Corynebacterium* group D2, encrusted in the mucus of the bladder. Magnification, $\times 100$.

inoculated with *Corynebacterium* group D2 and sacrificed after 12 days, 8 animals (33.3%) did not carry the bacteria that had been initially implanted; no bacteria were found in 5, and different microorganisms were found in 3 (*Proteus* sp. and *Streptococcus* group D). In the other 16 animals (66.6%), *Corynebacterium* group D2 remained, in 2 cases being associated with other bacteria (*Staphylococcus aureus* and *Streptococcus* group D). Only animals having *Corynebacterium* group D2 or other urea-splitting bacteria showed stones on the disks or encrusted in the mucosa of the bladder. In the second group, composed of 10 animals inoculated with *E. coli*, 2 rats expired 48 h after inoculation. Only one rat from this group showed a struvite stone, but *Proteus* sp. was isolated in association with *E. coli* in this animal. Figure 2 shows bladder stones formed on zinc disks

that had been dipped into a broth culture of *Corynebacterium* group D2, and Fig. 3 shows the gross appearance of a bladder with deposition of hard gritty material on the affected areas; these were definite bladder stones in some animals.

The infrared spectra of the stones and the NH_4MgPO_4 control resembled each other (Fig. 4). All spectra showed two deep absorbances at 1,000 and 2,900 cm^{-1} and two shallow ones at 1,430 and 1,600 cm^{-1} , although trace quantities of apatite [$\text{Ca}_5(\text{OH})(\text{PO}_4)_3$] were also present in some cases.

DISCUSSION

In the present paper, we have shown that *Corynebacterium* group D2 growing in human urine formed crystals of ammonium magnesium phosphate (struvite). Accompanied by the growth of cells, the ammonium concentration and pH increased and struvite crystals appeared after 24 h. The same results were obtained using *P. vulgaris* as has been previously reported with this organism (2, 9) and, more recently, with *Ureaplasma urealyticum* (7). Non-urea-splitting bacteria such as *E. coli* growing in human urine did not show any precipitate or crystals, and the ammonium concentration and pH, after 24 h of incubation, remained at the same levels as at the beginning of the experiment. Urine with no microorganisms, incubated under the same conditions, did not show any alteration in pH, ammonium concentration, or sediment.

The animal model showed that *Corynebacterium* group D2, inoculated on a zinc disk into rat bladders, is able to induce stone formation, as has already been shown with other bacteria (8, 10) including *U. urealyticum* (4). In our experiments *Corynebacterium* group D2 produced variable precipitates of struvite as well as encrusted cystitis, a condition previously reported to occur in human beings (6). Some differences exist between the in vivo results obtained with *Corynebacterium* group D2 and published findings using other urea-splitting bacteria such as *Proteus* sp. (1, 8, 10). *Corynebacterium* group D2 is a relatively fastidious microorganism and is probably less virulent than other gram-negative bacilli usually involved in urinary tract infections. Eight of the 24 rats inoculated with *Corynebacterium*

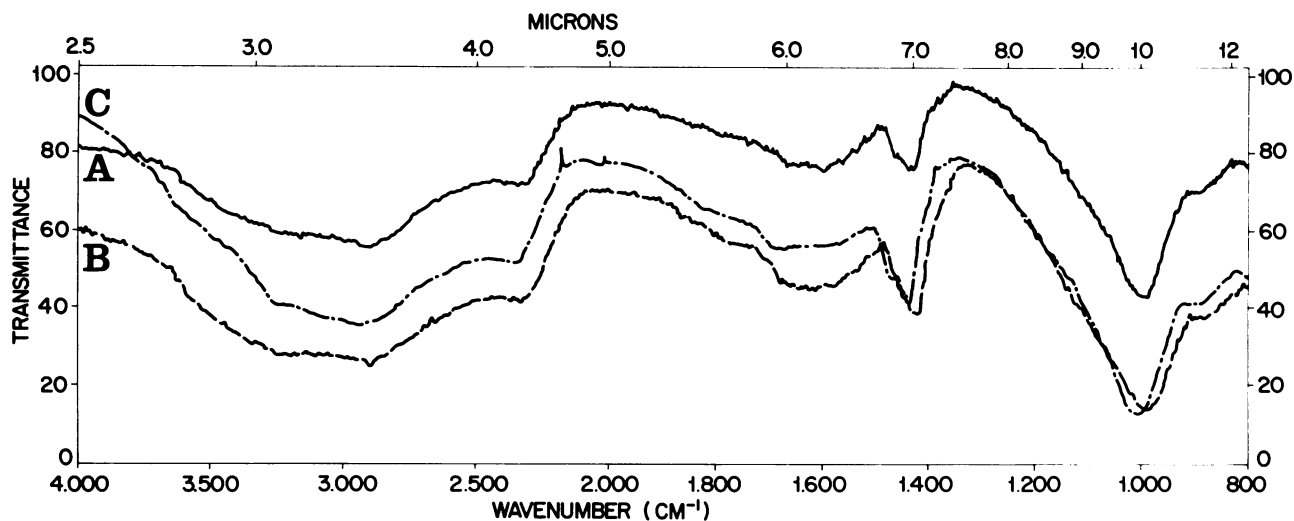


FIG. 4. Infrared spectra of urinary stones. The infrared spectra of two bladder stones formed by *Corynebacterium* group D2 (A and B) and NH_4MgPO_4 control (C) are shown.

group D2 were able to spontaneously eliminate these bacteria from the bladder, and there was no mortality in this group. In contrast, animals inoculated with *E. coli* maintained this microorganism in their bladders, and two rats died. Animal models using *Proteus* sp. have shown significantly high mortality (1, 10), and infection persisted in most of the surviving rats. At 1 to 3 weeks after inoculation, the infected surviving rats showed more struvite crystals than in our experiment with *Corynebacterium* group D2. In our group of animals inoculated with *Corynebacterium* group D2, there was a substitution by *Proteus* sp. in one animal, and this rat had the largest struvite stone observed in our experiments. In the group inoculated with *E. coli* there was a superinfection by *Proteus* sp. in one animal, which was the only one in this group showing a struvite stone. Animals with a negative culture or superinfection by a non-urea-splitting bacterium did not show any stones in their bladders.

In summary, *Corynebacterium* group D2 as well as other urease-positive bacteria have been isolated, mostly in pure culture, from patients with struvite urinary stones (6). In vitro studies have shown that this microorganism is capable of inducing the precipitation of such stones in human urine. The animal model has proved to be a quite reproducible experiment for the encrusted cystitis produced by *Corynebacterium* group D2 with formation of struvite stones. All these data strongly support most of Koch's postulates to recognize the involvement of *Corynebacterium* group D2 in the above-mentioned infections in humans.

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