大韓獸醫學會誌(1994) 第34권 第1號
Korean J Vet Res (1994) 34(1):99-105

Studies on the *Clostridium bovis* sp. nov., the predominant species isolated from the feces of Holstein cattle

Wan-kyu Lee

College of Veterinary Medicine, Chungbuk National University

(Received Jan 19, 1994)

홀스타인 젖소의 분변에서 우세균종으로 분리되는 새로운 *Clostridium bovis*에 관한 연구

이 완 규

충북대학교 수의과대학

(1994년 1월 19일 접수)

초 록 : 홀스타인 젖소의 분변에서 우세균종으로 분리되는 동정불능 *Clostridium*균주에 대해 당분해 성상검사, 생화학적 성상검사, G+C mol% 측정, 유사 균종과의 DNA-DNA hybridiazation 등을 조사한 결과, 기존의 *Clostridium* 균종과는 성상이 일치하지 않는 새로운 신균종(New species)임이 처음으로 밝혀져, 이 균주를 *Clostridium bovis*로 분류, 명명하였다.

이 *C. bovis*는 그램 양성, 운동성의 편성 혐기성 아포형성 간균이었으며, 당분해 성상은 arabinose, xylose, glucose, mannose, fructose, galactose, sucrose, maltose, cellobiose, lactose, trehalose, melibiose, raffinose, inulin, salicin을 분해하였다. Gas chromatography를 사용하여 PYFG broth로부터의 최종대사산물(End products)를 측정한 결과, 다량의 butyric, lactic acid와 소량의 acetic, succinic acid를 생산하였다. *C. bovis*의 Type strain은 Catt 66^{T}이며, G+C mol%는 26 mol%이었다.

Key word : *Clostridium bovis*, new species, taxonomy, cattle

Introduction

The genus *Clostridium* are widely distributed in intestinal tract of various domestic animals, and they are important members of normal intestinal microflora.[2,21] Although some species of *Clostridium; C.tetani*[7,31], *C. chauvoei*[14], *C. perfringens*[5,6,13], *C.novyi*[1], and *C. haemolyticum*[23,30] are causative agents of disease in cattle, the majority of intestinal clostridia are non-pathogenic or their possibility of pathogenecity are still not clear. Furthermore taxonomic and ecologic position of these normal intestinal clostridia in cattle are not cleary determined.

During the quantitative and qualitative studies on the fecal microflora of Holstein catte(1-7 years), unidentified frive strains were isolated. These strains were Gram-positive, motile, strict anaerobic sporeforming rods, and reclassified by their biochemical characteristics, G+C mol% and DNA-DNA molecular hybridization data. On the basis of the biochemical and nucleic acid data, the isolates sufficiently differed from the previously described *Clostridium* species, and warrant a new species, for which the name *Clostridium bovis* sp. nov. is proposed.

Materials and Methods

Bacterial strains : A total of 13 strains were used in this study. Five of these were isolated from the feces of apparently healthy Holstein cattle(N=5). *Clostridium sartagoforme* JCM 1413T, *C. celatum* ATCC 27791^T, *C. rectum* ATCC 25751^T, *C. butyricum* ATCC 19398^T, *C. difficile* ATCC 9689^T, *C. paraputrificum* JCM 1293^T, *C. tertium* JCM 7289^T, *C. gallinarum* WKCh5^T were also used as type strains.

Isolation and culture technique : Fresh feces were collected aseptically from the apparently healthy Holstein cattle. A 10-fold dilution was made with prereduced phosphate buffered saline[21,22] and divided into two parts. One was heated at 75℃ for 10 min. in a water bath and then plated onto each of four non-selective media[21,22]: modified Eggerth-Gagnon (EG) agar, glucose blood liver(BL) agar, Egg yolk agar[9] and Trypticase soy(TS) blood agar(BBL). EG, BL and Egg yolk agar plates were incubated at 37℃ for 48 hr in an anaerobic steel wool jar[21] filled with O_2 free CO_2 gas, while TS agar plates were incubated at 37℃ for 24 hr aerobically. The other part, a 10-fold dilution left unheated, was directly plated onto each of three selective media for Clostridia[21]: propionate oleandomycin(PO) agar, propionate novobiocin colimycin(PNC) agar and rifampicin colimycin neomycin(RCN) agar. PO, PNC and RCN agar consisted of 40.7g of EG agar(Eiken, Co.). 0.5g of tween 80 and the following selective agents(per liter); 15g of sodium propionate and 5mg of oleandomycin for PO agar; 15g of sodium propionate, 0.5ml of 1% novobiocin and 1ml of 1% colimycin for PNC agar; 0.5ml of 1% rifampicin, 1ml of 1% colimycin and 20ml of 1% neomycin for RCN agar. PO, PNC and RCN agar plates were incubated at 37℃ for 48 hr in an anaerobic steel wool jar filled with O_2 free Co_2 gas. Thereafter, strict anaerobic sporeforming strains were selected and carefully examined for their colony morphology, gram-reaction, spore formation and cell morphology. The isolates were stored on prereduced EGLF slants[22] at 4℃ until further tests for characterization. Transfers of cultures were made at intervals of 3 months.

Identification : Biochemical tests used for identification of genus *Clostridium* in this study were carbohydrate fermentation tests, starch hydrolysis, esculin hydrolysis, gas formation, H_2S production, indole production, nitrate reduction, motility, gelatin liquefaction, catalase, hemolysis, lecithinase, lipase and end products from PYFG broth[21].

Carbohydrate fermentation tests wre determined from 30 carbohydrates(Sigma Co.) The basal medium for the carbohydrate fermentation was peptone yeast extract broth supplemented with 4%(v/v) Fildes' peptic digest of horse blood, designated PYF broth. Each carbohydrate was added to PYF broth to give a final concentration of 0.5%(w/v). After inoculation, incubation was carried out at 37℃ for 7 days in an anaerobic chamber with an atmosphere of 90% N_2-10% CO_2. The final pH was measured directly in the culture tubes by combination glass electrodes[17]. Esculin and starch hydrolysis, gelatin liquefaction, lecithinase, catalase, and lipase production were tested by the methods described by Holdeman et al[9]. A modified SIM medium(Difco) was used for the production of H_2S. Formation of gas was detected in PYF solution supplemented wit 1%(w/v) glucose and 1.5%(w/v) Bacto agar(Difco). Indole production and nitrate reduction were determined with indole nitrate medium(BBL). Volatile and non-volatile fatty acids produced PYF broth supplemented with 1%(w/v) glucose(PYFG broth) were analyzed by the methods of Kaneuch et al[12], using a gas chromatography system(GC-4BMPF, Shimazu Ltd.)

Determination of guanine plus cytosine mol%(G+C mol%): Cells grown in EG broth were harvested in the logarithmic phase and washed twice with 0.15M NaCl-0.1M EDTA solution(pH 8.0). Deoxyribonucleic acid(DNA) was isolated and purified by modified procedures of Marmur[19] and Saito and Miura[26]. The guanine plus cytosine mol%(G+C mol%) of the purified DNA was determined by the thermal melting point(Tm) method[20] using a spectrophotometer equipped with Tm analysis system (DU-8B; Beckman Co. Ltd.). DNA from a *E. coli* strain(Type Ⅷ; Sigma) with a G+C content of 52.9 mol% was used as a reference.

DNA-DNA molecular hybridization : DNA homology studies were performed by the Sl nuclease

procedure as described by Johnson et al[10,11]. Sl nuclease digestions were conducted with 0.5U of Sl nuclease(Tokyo Kasei Ltd.). After incubation for 15 min. at 37℃, the remaining double-stranded DNA segments were precipitated with cold 10% trichloracetic acid and collected on membrane filters (type HA; Millipore Corp., Bedford, Mass.). The membranes were dried, and the radioactivity was measured in toluene-based scintillation fluid with a liquid scintillation counter(model 3330; Packard Instrument Co.).

Results

Colony and cell morphology are shown in Fig.1. After anaerobic incubation at 37℃ for 48 hr on EG agar plates, cells were straight or slightly curved rod-shaped and their size are 0.3-0.6μm by 1.2-3.0μm. Spores were subteriminal and oval shaped, and their size was 0.7-0.9μm by 0.8-1.7μm. Surface colonies are 3-8mm in diameter, and irregular, flat, undulate, rough, translucent and whitish gray.

Biochemical characteristics of these strains are shown in Table 1. All of the strains produced acid from arabinose, xylose, glucose, mannose, fructose, galactose, sucrose, maltose, cellobiose, lactose, trehalose, melibiose, raffinose, inulin and salicin, but not from rhamnose, sorbose, ribose, melezitose, starch, glycogen, esculin, mannitol, sorbitol, erythritol, inositol, dulcitol and glycerol. Gas production and motility were positive. Esculin is hydrolyzed but starch is not. The following characteristics are negative: H_2S and indole production, lecithinase, lipase, catalase, nitrate reduction, gelatin liquefaction and hemolysis. End products from PYFG broth were large amounts of butyric and lactic acids, and trace amounts of acetic and succinic acids.

Table 2 presents the G+C mol% of DNA from these isolates and the DNA homology values among the other representative speceies of *Clostridium*. The DNAs from these isolates were highly homologous to each other and had little or no homology with the DNAs of each other related *Clostridium* species.

The genetic data and biochemical differences clearly indicate that the isolates represent a new *Clostridium* species, for which I proposed the name *Clostridium gallinarum*.

Description of *Clostridium bovis* sp. nov. : *Clostridium bovis*(bo'vis. L.n. *bos* cow; L.gen.n.*bovis* of cow). Strictly anaerobic, motile, sporeforming rods. Cells are straight or slightly curved rod-shaped and their size are 0.3-0.6μm by 1.2-3.0μm. Spores were subterminal and oval shaped. Surface colonies are irregular, flat, undulate, rough, translucent and whitish gray. Acid is produced from arabinose, xylose, glucose, mannose, fructose, galactose, sucrose, maltose, cellobiose, lactose, trehalose, melibiose, raffinose, inulin and salicin. Esculin hydrolysis, gas production and motility are positive. The following characteristics are negative: production of H_2S, indole production, lecithinase, lipase, catalase, nitrate reduction, gelatin liquefaction and hemolysis. End products from PYFG broth are aBLs. Isolated from the feces of cattle. The type strain is Catt 66^T strain. The G+C content of DNA from type strain is 26 mol%.

Table 4 shows the biochemical characteristics that help to differentiate *C. bovis* from other related clostridial species: *C. gallinarum. C. celatum, C. rectum, C. sartagoforme* and *C. paraputrificum*.

Discussion

Identification of a clostridial isolates at the species level is very difficult. Generally speaking, the taxonomy of the genus *Clostridium* is still in an unsatisfactory state. Definitive identification of clostridia requires a description of their cell and colony morphology. Gram reaction, spore determination, aerotolerance, acid production from carbohydrates, other biochemical characteristics, and determination of volatile and non-vloatile fatty acids[9,21,25]. Testes for animal pathogenicity and toxin neutralization may also be performed. However, taxonomic criteria such as DNA base ratio(G+C mol%), DNA-DNA hybridization capacity, cell wall composition[3,8,27] and numerical analysis[15,24] have not been applied widely to clostridia. Some clarification of

Table 1. Biochemical characteristics of *Clostridium bovis* strains and its type strain

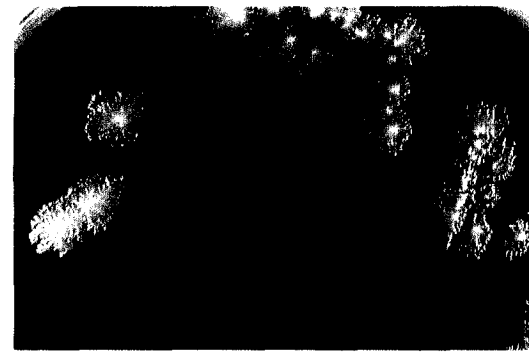
Characteristics	Results for *C. bovis*[a]	
	4 strains	Type strains(Catt 66[T])
Final pH in PYFG	5.0-5.3	5.0
Starch hydrolysis	−	−
Esculin hydrolysis	+	+
Gas formation	+	+
H_2S Production	−	−
Indole production	−	−
Nitrate reduction	−	−
Motility	+	+
Gelatin liquefaction	−	−
Catalase	−	−
Hemolysis	−	−
Lecithinase	−	−
Lipase	−	−
Products from PYFG broth[b]	aBLs	aBLs
G+C mol %	26-27	26
Acid from		
Arabinose	+	+
Xylose	+	+
Rhamnose	−	−
Sorbose	−	−
Ribose	−	−
Glucose	+	+
Mannose	+	+
Fructose	+	+
Galactose	+	+
Sucrose	+	+
Maltose	+	+
Cellobiose	+	+
Lactose	+	+
Trehalose	+	+
Melibiose	+	+
Raffinose	+	+
Melezitose	−	−
Starch	−	−
Glycogen	−	−
Inulin	+	+
Glycerol	−	−
Mannitol	−/w	−
Sorbitol	−	−
Erythritol	−	−
Inositol	−	−
Dulcitol	−	−
Esculin	−	−
Salicin	+	+
Amygdalin	w	w

[a] +, positive for 90-100% of strains(pH below 5.5); -, negative for 90-100% of strains; w, weak reaction(pH 5.5-5.9)

[b] a, acetic acid; b, butyric acid; l, lactic acid; s, succinic acid. Capital letters indicate at least 1 meq/100ml of culture; small letters indicate less than 1 meq/100ml.

relationship among small groups of species has been achived but the integrity of the genus *Clostridium*[10] and the larger groups within it remains a problem.

Early classification of organisms in the genus



(A)

(B)

Fig 1. Colonial and cellurar morphology of the type strain Catt 66[T] of *C. bovis*.
A: Surface colonies on the EG agar incubated anaerobically at 37℃ for 48hr. Bar represents 10mm.
B: Gram stained cells from EG agar incubated anaerobically for 48hr. Bar represents 10㎛.

Clostridium had mainly been undertaken on their pathogenicity rather than on their general biological characteristics. Because of their significance on medical and veterinary science, toxin producing species such as *C. tetani, C. botulinum*[4,28,29], *C. perfringens, C. septicum*[16] and *C. difficile*[18] have been fully investigated. However, the majority of normal intestinal clostridia in various domestic animals are considered to be non pathogenic or their mechanism of pathogenicity, if existent, is still not clear. Furthermore, taxonomical positions of these normal intestinal clostridia in humans and animals have still not been clarified. Further studies will be necessary to clarify the taxonomic and ecologic positions of these intestinal clostridia in veterinary medicine.

Table 2. DNA-DNA hybridization result for *Clostridium bovis* strains and type strains of related other *Clostridium* species

Species and strains	G+C content (mol %)	Relative binding ratio(%) with H^3 labeled DNA from:				
		C. bovis			*C. gallinarum*	*C. sartagoforme*
		Catt66^T	Catt112	Catt53	WKCh5^S	JCM1413^T
C. bovis						
Catt 66^T	26	100	80	88	12	3
Catt 112	27	70	100	91	10	1
Catt 53	27	85	94	100	8	1
Catt 8	26	95	92	93	11	2
Catt 75	26	93	90	92	10	2
C. gallinarum WKCh5^T	27	10	8	7	100	3
C. celatum ATCC 27791^T	NT[a]	3	2	3	2	5
C. rectum ATCC 25751^T	26	1	1	2	0	0
C. sartagoform JCM 1413^T	28	3	4	2	0	100
C. butyricum ATCC 19398^T	28	5	4	3	1	2
C. difficile ATCC 9689^T	28	2	2	3	2	0
C. paraputrificum JCM 1293^T	27	1	2	2	0	0
C. tertium JCM 7289^T	26	2	1	3	0	0

[a] NT, Not tested

Table 3. Characteristics that help to differentiate *Clostridium bovis* from other related *Clostridium* species

Characteristics	Results for [a]					
	C. bovis	*C. gallinarum*	*C. celatum*	*C. rectum*	*C. sartagoforme*	*C. paraputrificum*
Acid from:						
Arabinose	+	+	−	−	−	−
Rhamnose	−	+	−	−	−/w	−
Melibiose	+	+	−	−	+	−
Melezitose	−	+	−	−	−/w	−
Inulin	+	+	−	−	+	−
Sorbitol	−	+/w	−	−	−	−
End products from PYFG broth [b]	aBLs	aBs	AFb2(s)	Bapv	BAF(L)	BAL(sf)

[a] +, positive for 90-100% of strains(pH below 5.5); -. negative for 90-100% of strains; w, weak reaction(pH 5.5-5.9)

[b] a, acetic acid; b, butyric acid; l, lactic acid; s, succinic acid; f, formic acid; p, propionic acid; v, valeric acid; 2, ethanol. Capital letters indicate at least 1 meq/100ml of culture; small letters indicate less than 1 meq/100ml. Parentheses indicate products of some strains.

Summary

Clotridium bovis sp. nov, is described on the basis of 5 strains isolated from the feces of Holstein cattle. The isolate are gram-positive, motile, strict anaerobic sporeforming rods. They differ from all the validly described related species of the genus *Clostridium* in carbohydrate fermentation pattern, G+C mol% and DNA homologies. Acid is produced from arabinose, xylose, glucose, mannose, fructose, galactose, sucrose, maltose, cellobiose, lactose, trehalose, melibiose, raffinose, inulin and salicin. Major end products in PYFG broth are large amounts of butyric acid and lactic acid, and trace amounts of acetic and succinic acids. The G+C mol% of DNA from the type strain is 26 mol%. The type strain of *Clostridium bovis* is Catt 66^T strain.

References

1. Batty I, Buntain D, Walker PD. *Clostridium oedematiens:* A cause of sudden death in sheep, cattle and pigs. *Vet Rec* 1964; 76:1115-1116.
2. Cato EP, George WL, Finegold SM. Genus *Clostridium Prazmowski* 1880, 23[AL], Bergey's manual of systematic bacteriology, *vol.* 2. 1986; 1141-1200.
3. Cummins CS, Johnson JL. Taxonomy of the clostridia: wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid-producing clostridia. *J Gen Microbiol* 1971; 67:33-46.
4. Davies AB, Roberts TA, Bradchaw PR. Probable botulism in calves. *Vet Rec* 1974; 94:412-414.
5. Frank FW. *Clostridium perfringens* type B from enterotoxemia in young ruminants. *Am J Vet Res* 1956; 17:492-494.
6. Griner LA, Bracken FK. *Clostridium perfringens*(type C) in acute hemorrhagic enteritis of calves. *J Am Vet Med Assoc* 1953; 125:99-102.
7. Herd RP, and Riches WR. An outbreak of tetanus in cattle. *Aust Vet J* 1964; 40:356-357.
8. Hobbs G, Hardy R, Mackie PR. Characterization of Clostridium species by means of their lipids. *J Gen Microbiol* 1971; 68:2-3.
9. Holdeman LV, Cato EP, Moore WEC. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University. Blacksburg. 1977
10. Johnson JL, Francis B. The taxomomy of the clostridia: ribosomal homologies among the species. *J Gen Microbiol* 1975; 88:229-24-.
11. Johnson JL, Phelps CF, [illegible] CS, London J, Gasser F. Taxonomy of [illegible] *cillus acidophilus* group. *Int J Syst* [illegible] *acterio*[illegible] 0:53-68.
12. Kaneuchi C, Watanabe K, [illegible]oda A, Benno Y, Mitsuoka K. Taxonomic study of *Bacteroides clostridiiformis* subsp. *clostridiiformis*(Burri and Ankersmit) Holdeman and Moore and of related organisms; proposal of *Clostridium clostridiiformis*(Burri and Ankersmit) comb. nov. and *Clostridium symbiosum* (Stevens) comb. nov. *Int J Syst Bacteriol* 1976; 26: 195-204.
13. Kennedy KK, Norris SJ, Bechenhauer WH, White RG. Vaccination of cattle [illegible]heep with combined *Clostridium perfringens* ty[illegible] D toxoid. *Am J Vet Res* 1977; 38:1515-1517.
14. Kerry JB. A note on the occurrence of *Clostridium chauvoei* in the spleen and livers of normal cattle. *Vet Rec* 1964; 76:396-397.
15. Kiritani K, Mitsui N, Nakamura S, Nishida S. Numerical taxonomy of *Clostridium botulinum* and *Clostridium sporogenes* strains and their susceptibilities to induced lysins and to mitomycin c. *Jap J Microbiol* 1973; 178:361-372.
16. Koransky JR, Stargel MD, Dowell VR, Jr, *Clostridium septicum* bacteremia: Its clinical significance. *Am J Med* 1979; 66:63-66.
17. Lee WK, Fujisawa T, Kawamura S, Itoh K, Mitsuoka T. *Clostridium intestinalis* sp. nov., an aerotolerant species isolated from the feces of cattle and pigs. *Int J Syst Bacteriol* 1989; 39:334-336.
18. Lyerly DM, Krivan HC, Wilkins TD. *Clostridium difficile:* Its Disease and Toxins. *Clin Microbiol Rev* 1988; 1:1-18.
19. Marmur J. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 1961; 3:208-218.
20. Marmur J, Doty P. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* 1962; 5:109-118.
21. Mitsuoka T. The World of Anaerobic Bacteria; A Color Atlas of Anaerobic Bacteria, 1 st ed., Sobun Press, Tokyo 1980.
22. Mitsuoka T, Sega T, Yamamoto S. Eine verbesserte Methodik der qualitativen and quantitatven Analyse der Darmflora von Menschen und Tieren. *Zent Bakt Para Hyg 1 Abt Orig* 1965; 195:455-469.
23. Nakamura S, Kimura I, Yamakawa K, Nishida S. Taxonomic relationshipa among *Clostridium novyi* types A and B, *Clostridium hlaemolyticum* and *Clostridium botulinum* type C. *J Gen Microbiol* 1983; 139:1473-1479.
24. Nakamura S, Shimamura T, Hayase M, Nishida S. Numerical taxonomy of saccharolytic Clostridia, particulary *Clostridium perfringens*-like strains: description of *Clostridium absonum* sp. n. and *Clostridium paraperfringens. Int J Syst Bacteriol* 1973; 23:419-429.
25. Princewall TJT, Agba MI. Examination of bovine

feces for the isolation and identification of Clostridium species. *J Appl Bacteriol* 1982; 52:97-102.

26. Saito H, Miura K. Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochem Biophys Acta* 1963; 72:642-644.
27. Schleifer KH, Kandler O.Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* 1972; 36:407-477.
28. Smart JL, Roberts TA. Bovine botulism, *Vet Rec* 1977; 101:201-202.
29. Smith LDS. Botulism, the organism, its toxins, the disease. Springfield, Illinois: Charles C. Thomas. 1977; 1-126.
30. Smith L, Jasmin AM. The recovery of *Clostridium olyticum* from the livers and kidneys of apparently normal cattle. *J Am Vet Med Assoc* 1956; 129:68-71.
31. Wallis AS. Some observation on the epidemiology of tetanus in cattle. *Vet Rec* 1963; 75:188-191.