International Journal of Microbiological Research 3 (2): 128-132, 2012
ISSN 2079-2093

DOI: 10.5829/idosi.ijmr.2012.3.2.62104

Colistin Sulphate as a Substitute of Thallium Acetate in Culture Media for Isolation of Mycoplasma from Cattle Diseased by Mycoplasmosis

[1,2]M.H. Yassin, [1]A.A. Al-Humiany and [3]A.M.A. Mansour

[1]Departement of Medical Microbiology,
Faculty of Applied Medical Science, Tarabah, Taif University, KSA
[2]Departement of Reproductive Diseases, ARRI, Giza, Egypt
[3]Departement of Medical Laboratory,
Faculty of Applied Medical Science, Tarabah, Taif University, KSA

Abstract: Mycoplasmas cause several diseases in cattle as pneumonia in calves and mastitis in cows and also reproductive disorders, abortions, arthritis and conjunctivitis. The difficulty of culturing mycoplasmas *in vitro* is a major obstacle to research and laboratory diagnosis of these fastidious organisms. A total of 120 preputial swabs, 70 vaginal swabs and 65 milk samples were collected from cattle at several farms (in which mycoplasma infection was suspected) suffering from mastitis and/or reproductive disorders and artificial insemination centers for isolation of Mycoplasma and Ureaplasma, the recovery rates of Mycoplasma and Ureaplasma from the prepuce of bulls were (12.5% and 6.6% respectively) in addition to the isolation rate from vagina of cows were (5.7% and 1.4% respectively) while in milk the isolation rate of Mycoplasma was 3% and Ureaplasma was not isolated from milk samples, *Mycoplasma bovigentalium* was the most frequent species isolated (46.7 %) followed by *Ureaplasma diversum* (30%) and *Mycoplasma bovis* (23.3%). Thallium acetate is commonly used in the laboratories as a selective element against Gram-negative bacteria in selective media for the detection of mollicutes. Thallium acetate is enormously toxic and because it is not biodegradable its accumulation in the environment presents a very serious problem also Ureaplasma species and some mycoplasmas are very sensitive to thallium acetate and must be omitted from the media. The results of analysis of tests with six mycoplasma species are presented here (*Mycoplasma bovis, Mycoplasma bovigentalium, Mycoplasma arginini, Mycoplasma canis, Mycoplasma bovirhinis and Ureaplasma diversum*). The medium containing colistin sulphate appeared to be a useful substitute of thallium acetate for the detection and culture of the species tested in these experiments.

Key words: Mycoplasma · Cattle · Thallium Acetate · Colistin Sulphate

INTRODUCTION

Mycoplasma are the smallest free-living bacteria. They range from 0.2-0.8 micrometers and thus can pass through some bacterial filters. They have the smallest genome size and, as a result, lack many metabolic pathways and require complex media for their isolation. A characteristic feature that distinguishes mycoplasmas from other bacteria is the lack of a cell wall. Thus, they can assume multiple shapes including round, pear shaped and even filamentous, also without a cell wall, they are not affected by many common antibiotics such as penicillin or other beta-lactam antibiotics that target cell wall synthesis [1].

Mycoplasma cause several diseases in cattle as pneumonia in calves and mastitis in cows also reproductive disorders, abortions, arthritis and conjunctivitis [2-4].

The difficulty of culturing mycoplasmas *in vitro* is a major obstacle to research and laboratory diagnosis of these fastidious organisms and it is highly likely that many more mycoplasmas exist in nature but have not yet

Corresponding Author: M.H. Yassin, Departement of Medical Microbiology,
Faculty of Applied Medical Science, Tarabah, Taif University, KSA.

been isolated, despite great efforts over many years including the introduction of PCR, in addition, many isolated mycoplasmas still grow very poorly even on the best mycoplasma media [5, 6].

Since Edward [7] proposed the addition of thallium acetate to a selective media for the detection of mollicutes this chemical compound is commonly used in the laboratories. Thallium acetate offers the advantage that a large number of mycoplasmas and acholeplasmas can grow in its presence at concentration of 1 mg/ml. The advantage of using this chemical compound as a selective element against Gram-negative bacteria is bound to some inconveniences: Thallium acetate is enormously toxic and because it is not biodegradable its accumulation in the environment presents a very serious problems [8], also Ureaplasma species and some mycoplasmas are very sensitive to thallium acetate and must be omitted from the media [9, 10].

Colistin is an antibiotic of the polymyxin group produced by *Bacillus polymyxa* var. colistinus. It was discovered by Koyama *et al*. [11]. The polymyxins are cyclic lipopeptides; chemically, colistin is a strong organic base, used clinically in the form of its water soluble sulphate and methane sulphonate salts. It is a bactericide with activity against Gram negative enterobacteria and possess a rapid onset of action [12].

The aims of this study were to investigate the possibility of addition of colistin sulphate as a substitute of thallium acetate for inhibition of Gram-negative bacteria in selective culture media for isolation and identification of bovine mycoplasmas and study the role of mycoplasma in production of diseases in cattle.

MATERIALS AND METHODS

Samples: A total of 120 preputial swabs, 70 vaginal swabs and 65 milk samples were collected from cattle at several farms (in which mycoplasma infection was suspected) suffering from mastitis and/or reproductive disorders and artificial insemination centers for isolation of Mycoplasma and Ureaplasma.

Culture Procedure

Mycoplasma: The samples were cultured simultaneously by direct plating on mycoplasma agar and also inoculated in the broth media, the latter was subsequently plated after 3 days of incubation and then after 3 additional days. Broth and agar plates were incubated at 37°C and agar plates were incubated under 10% CO^2 tension. The incubated plates were examined microscopically for the characteristic fried egg appearance of mycoplasma colonies after 48 hours of incubation and every other day up to 10 days [13].

Ureaplasma: A serial ten fold dilution of original samples was done in Ureaplasma broth medium. Original and diluted samples were incubated aerobically at 37°C when the color changed from yellow to red without turbidity started from down upward, the changed broth was sub-cultured on Ureaplasma agar plates as soon as possible, agar plates were incubated under 10% CO_2 tension The incubated plates were examined microscopically for the characteristic Ureaplasma colonies [13].

Genus Determination of *Mycoplasma and Ureaplasma* Isolates: It was performed using digitonin sensitivity test [13].

Biochemical Characterization of *Mycoplasma* Isolates: It was performed by glucose fermentation and arginine deamination [14].

Serological Identification of *Mycoplasma* Isolates: The isolated strains were identified by the growth inhibition (GI) test and metabolic inhibition test (MI) test [15].

Media Comparison: For this study for mycoplasma media; bacto-brain heart infusion agar or broth supplemented with 20% horse serum, 10% yeast extract, 0.02% DNA, 1000IU/ml penicillin G sodium and 1% of 10% thallium acetate [14] in the medium with colistin an amount of 37.5mg/L colistin sulphate takes the place of thallium acetate. While for Ureaplasma PPLO broth (U9) pH6 supplemented with 20% horse serum. 10% yeast extract, 4% of urea 25% solution, 1000IU/ml penicillin G sodium, 0.2% phenol red and 37.5mg/L colistin sulphate.

Strains and Culture Conditions: Reference strains of *Mycoplasma bovis*, *Mycoplasma bovigentalium*, *Mycoplasma arginini, Mycoplasma canis* and *Mycoplasma bovirhinis* were obtained from prof. Nicholas Robin Veterinary Laboratory Agency UK and Field isolates of *Mycoplasma bovis, Mycoplasma bovigentalium* and *Ureaplasma diversum*.

Each strain was prepared in saline and inoculated into broth of the two media compositions. After (two days for Mycoplasmas and 18 hours for Ureaplasma) incubation

subculture of each dilution was done in the respective agar plates. Microscopical observation and counting of colonies was carried out in the five days following the inoculation, the experiment was repeated six times.

RESULTS AND DISCUSSION

The present investigation (Table 1) showed that the recovery rates of Mycoplasma and Ureaplasma from the prepuce of bulls were (12.5% and 6.6% respectively) in addition to the isolation rate from vagina of cows were (5.7% and 1.4% respectively) while in milk the isolation rate of Mycoplasma was 3% and Ureaplasma was not isolated from milk samples.

Mycoplasmas and ureaplasmas have been isolated more frequently from the genital tract of male than from female [16].

Genital mycoplasmosis in bulls generally has not been associated with any particular clinical syndrome and the infection seemed in apparent but the possibility of its transfer to the female genital tract during coitus or through artificial insemination could not be ruled out. These results are nearly similar to some investigators [17-19].

Identification of Mycoplasma and Ureaplasma isolates (Table 2) reported that *Mycoplasma bovigentalium* was the most frequent species isolated (46.7 %) followed by *Ureaplasma diversum* (30%) and *Mycoplasma bovis* (23.3%).

M. bovigenitalium, is mainly found in the reproductive tract of cattle with reduced fertility, endometritis and granular vulvitis also*, M. bovigenitalium* is commonly found in semen samples or sheath washings from cattle. *M. bovigenitalium* have been detected more frequently in cattle with reproductive disorders than in healthy cattle [20-22].

M. bovis is widely spread within the bovine population in enzootically infected areas. The infection is usually introduced to *M. bovis*-free herds by clinically healthy calves or young cattle shedding the mycoplasmas and, once established on multi-age sites, it becomes very difficult to eradicate. [23]. Infected cattle shed the mycoplasma for many months and even years, contact animals become infected via the respiratory tract, the teat canal or genital tract; artificial insemination with infected semen is another common route. Infection of the prepuce or urethra by *M. bovis* leads to an ascending infection of the testes, causing orchitis, vesiculitis, decrease of semen quality and ultimately shedding in the semen [24, 25].

Cases of mycoplasma mastitis often have the following characteristics; they are non-responsive to antibiotics and anti-inflammatory drugs, there is decreased milk production in recovered cows, high somatic cell counts, atrophy of affected quarters and spread of infection from quarter to quarter. Abortions may also occur, with isolation of *M. bovis* from aborted tissues [26, 1, 4].

Ureaplasma diversum has also been associated with sporadic cases of epidydimitis, orchitis, urethritis and seminal vesiculitis, leading to pain on ejaculation [27-29].

Mycoplasma may impair the motility of spermatozoa, experimental infections with artificially contaminated semen have shown that it can be isolated easily from washed embryos, where it forms a close association with

Table 1: Isolation of Mycoplasma and Ureaplasma from collected cattle samples

		Mycoplasma (M)		*Ureaplasma* (U)		*Mixed infection* (M&U)	
		+Ve No.	%	+Ve No.	%	+Ve No.	%
Prepuce	120	15	12.5	8	6.6	5	4.1
Vagina	70	4	5.7	1	1.4	1	1.4
Milk	65	2	3	-	-	-	-
Total	255	21	8.2	9	3.5	6	2.3

Table 2: Identification of Mycoplasma and Ureaplasma isolates from cattle.

		M. bovis		*M. bovigentalium*		*U. diversum*	
		+Ve No.	%	+Ve No.	%	+Ve No.	%
Prepuce	23	4	17.4	11	47.8	8	34.8
Vagina	5	1	20.0	3	60.0	1	20.0
Milk	2	2	100.0	-	-	-	-
Total	30	7	23.3	14	46.7	9	30.0

Table 3: Statistical comparison of the growth in the two agar media performed by ANOVA

	Mean of cfu/agar plate with thallium acetate	Mean of cfu/agar plate with colistin sulphate
M. bovis	3.667±0.557	3.333±0.447
M. bovigentalium	4.333±0.557	3.667±0.557
M. arginini	4.667±0.919	5.000±0.365
M. canis	3.333±0.760	3.000±0.365
M. bovirhinis	2.333±0.557	3.000±0.365
U. diversum	0.000 ± 0.000^{A}	2.667 ± 0.211^{B}

Means in the same row with different superscripts are significantly different at least at P<0.05

the surface of the zona pellucida, intact embryos and sperm cells attachment of the mycoplasma makes it difficult to eliminate with approved antibiotics [30, 31].

For this study we have used two agar media containing either colistin sulphate or thallium acetate for culturing the species of *Mycoplasma bovis, Mycoplasma bovigentalium, Mycoplasma arginini, Mycoplasma canis, Mycoplasma bovirhinis* and *Ureaplasma diversum* (Table 3) and the results indicated that except for *Ureaplasma diversum* there was no significant difference between the two agar media.

Significant difference (P<0.05) was found between the growths obtained in the cases of culture of *Ureaplasma diversum*. This species grows better into broth with colistin sulphate, but did not grow in media with thallium.

Thallium salts being colorless and odorless have been used for homicidal purposes and for illegal abortion, the diagnosis of thallium poisoning is not very easy and requires chemical analysis to confirm it [32] Thallium is more acutely toxic to mammals and it is absorbed through skin and mucous membranes, is widely distributed throughout the body and accumulates in bones, renal medulla and, eventually, in the central nervous system [33]. Ingestion large amounts of thallium over a short time have reported vomation, diarrhea, Temporary hair loss and effects on the nervous system, lungs, heart, liver and kidneys. It has caused death [34, 35].

It can be concluded that the use of this substitute is advantageous because it avoids the dangers of toxicity and contributes to reduce the pollution of the environment related to thallium acetate.

REFERENCES

1. Nicholas, N., R. Ayling and L. McAuliffe, 2008. Diseases caused by Mycoplasma bovis in Mycoplasma Diseases of Ruminants. Cabi, pp: 134-153.
2. Ruhnki, H.L., N.C. Palmer, P.A. Doig and R.B. Miller, 1984. Bovine abortion and neonatal death associated with Ureaplasma diversum. Theriogenol., 21: 295-301.
3. Caswel, J.L. and M. Archambault, 2009. Mycoplasma bovis pneumonia in cattle. Anim Health Res. Rev., 8: 161-186.
4. Maunsell, F.P., A.R. Woolums, D. Francoz, R.F. Rosenbusch, D.L. Step, D.J. Wilson and E.D. Janzen, 2011. Mycoplasma bovis Infections in Cattle. J. Vet. Intern. Med., 25: 772-783.
5. Razin, S., 1994. DNA probes and PCR in diagnosis of mycoplasma infections. Molecular and Cellular Probes., 8: 497-511.
6. Razin, S., D. Yogev and Y. Naot, 1998. Molecular biology and pathogenicity of mycoplasmas. Microbiology and Molecular Biology Reviews, 62: 1094-1156.
7. Edward, D.G., 1947. A selective medium for pleuropneumonia-like organisms. J. Gen. Microbiol., 1: 238-243.
8. Angulo, A.L., M.V. Jacobs, E.L. Van Damme, A.M. Akkermans and I.K. Jan Brugman, 1983. Colistin sulfate as a suitable substitute of thallium acetate in culture media intended for mycoplasma detection and culture. Biologicals, 31: 161-163.
9. Ruys, A.C., D. Herderschee and J. Waldman, 1967. Isolation and propagation of mycoplasma. Ann. NY Acad. Sci., 143: 390-393.
10. Tully, J.G., 1983. Bacterial and fungal inhibitors in mycoplasma culture media. In: Methods in mycoplasmology Eds., S. Razin, J.G. Tully, New York: Academic Press, 1: 205-209.
11. Koyama, Y., A. Kurosasa, A. Tsuchiya and K. Takakuta,1950.A new antibiotic ‘‘colistin” produced by spore-forming soil bacteria. J. Antibiotics, 3: 457-458.

12. Guyonnet, J., B. Manco, L. Baduel, V. Kaltsatos, M.H.F.S. Aliabadi and P. Lees, 2010. Determination of a dosage regimen of colistin by pharmacokinetic/ pharmacodynamic integration and modeling for treatment of G.I.T. disease in pigs Research in Veterinary Sci., 88: 307-314.
13. Ruhnke, H.L. and S. Rosendal, 1989. Useful protocols for diagnosis of animal mycoplasmas. WAVLD Mycoplasma Workshop Vet. Microbiol. and Immunol. Dept. Ontario Vet. Collage, Guelph, Ontario, Canada NIC2W1.
14. Erno, H. and L. Stipkovits, 1973. Bovine mycoplasma: cultural and biochemical studies. Acta Vet. Scand., 14: 450-463.
15. Clyde, W.A., 1983. Growth inhibition test. Methods on Mycoplasmology Vol. 1,405 Academic Press, New York.
16. Afshar, A., 1975. Diseases of bovine reproduction associated with Mycoplasma infections. Vet. Bull., 45: 211-216.
17. Trichard, C.J.V. and E.P. Jacobsz, 1985. Mycoplasmas recovered from bovine genitalia, aborted fetuses and placentas in the Republic of South Africa. Onderstepoort J. Vet. Res., 52(2): 105-110.
18. Clyde, A. and D.A.M. Kirkbride, 1987. Mycoplasma, Ureaplasma and Acholeplasma infection of bovine genitalia. Vet. Clinic.North America Food Anima1 Practice, 3: 575-591.
19. Pathak, R.C. and D.N. Garg, 1988. Immunobiology of bovine genital mycoplasmas. Prog. Vet. Microbiol. Immunol., 4: 218-345.
20. Ruhnke, H.L., 1994. Mycoplasmas associated with bovine genital tract infection. Mycoplasmosis in animal Laboratory Diagnosis eds. W. Howard, R.F. Rosenbush and L.H. Laureman, pp: 57-62.
21. Ashwani, K., D.N. Garg and A. Kumar, 1996. Detection of serum antibodies to Mycoplasma bovis and M. bovigenitalium in mastitic cows and buffaloes by ELISA, indirect haemagglutination test and agar-gel-immunoprecipitation test. Indian Veterinary Journal, 73: 603-606.
22. Garg, D.N., P.K. Kapoor and Y. Singh, 1999. Detection of mycoplasmal antibodies in bovines with reproductive disorders. Indian Journal of Comparative Microbiology, Immunology and Infectious Diseases, 20: 32-35.
23. Gourlay, R.N., I.H. Thomas and S.G. Wyld, 1989. Increased severity of calf pneumonia associated with the appearance of Mycoplasma bovis in a rearing herd. Veterinary Record, 124: 420-422.
24. Pfützner, H., 1990. Epizootiology of the Mycoplasma bovis infection of cattle. Zentralblatt fur Bakteriologie Supplement, 20: 394-399.
25. Kreusel, S., H. Bocklisch, H. Pfützner, A. Brys, R. Leirer and U. Ziegenhals, 1989.Experimentelle infektionen von bullen mit Mycoplasma bovis und Mycoplasma bovigenitalium.Archiv fur Experimentelle Veterinarmedizin, 43: 705-712.
26. Jasper, D.E., 1981. Bovine Mycoplasma mastitis. Adv. Vet. Sci. Comp. Med., 25: 121-157.
27. Ball, H.J., 1990. Use of bovine sheath washings for screening for mycoplasmas. Veterinary Record, 127: 16-17.
28. Gummow, B., G.P. Staley and J.J. Gouws, 1992. The diagnosis and treatment of bovine genital ureaplasmosis: a case study. Journal of the South African Veterinary Association, 63: 128-131.
29. Cardoso, M.V., A. Blanchard, S. Ferris, R. Verlengia, J. Timenetsky and J. Cunha, 2000. Detection of Ureaplasma diversum in cattle using a newly developed PCR-based detection assay. Veterinary Microbiol., 72: 241-250.
30. Ross, R.F., 1993. Mycoplasmas - animal pathogens. In: I. Kahane and A. Adoni, (eds) Rapid Diagnosis of Mycoplasmas. Plenum Press, New York, pp: 69-110.
31. Bielanski, A., J.D. Evenish and B. Phipps Todd, 2000. Effect of Mycoplasma bovis and Mycoplasma bovigenitalium in semen on fertilization and association with in vitro produced morula and blastocyst stage embryos. Theriogenol., 53(6): 1213-1223.
32. Kazantzis, G., 2000. Thallium in the environment and health effects. Environ. Geochem Health, 22: 275-280.
33. Cheam, V., 2001. Thallium contamination of water in Canada. Water Qual Res. J. Can., 36(4): 851-877.
34. Enviro Tools, 2002. Factsheets on Thallium. (Available at http:// www.envirotools.org/ factsheets/ contaminants/thallium.shtml).
35. John Peter, A.L. and T. Viraraghavan, 2005. Thallium: a review of public health and environmental concerns. Environ. International, 31: 493-501.