ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 6, Suppl 3, 2013

ISSN - 0974-2441

Research Article

IDENTIFICATION AND CHARACTERIZATION OF *LACTOCOCCUS GARVIEAE* AND ANTIMICROBIAL ACTIVITY OF ITS BACTERIOCIN ISOLATED FROM COW'S MILK

DODAMANI SUNEEL, KALIWAL BASAPPA *

Department of Biotechnology and Microbiology, Karnatak University, Dharwad, Karnataka, India. Email: b_kaliwal@yahoo.com

Received: 9 May 2013, Revised and Accepted: 18 July 2013

ABSTRACT

This study was conducted to isolate lactic acid bacteria (LAB) from cow's milk and characterize through morphological, physiological, biochemical, carbohydrate fermentation and its antimicrobial property. The bacteria were isolated by serial dilution of milk samples and confirmed through biochemical, physiological and 16s rRNA sequencing. The antibiotic susceptibility test of bacteria, antimicrobial test of bacteriocin by agar well diffusion assay was carried out. A total of 12 LAB has been isolated among them one isolate which showed good antimicrobial activity against Gram-positive bacteria viz., Staphylococcus aureus, Bacillus subtilis and Bacillus cereus and gram-negative bacteria viz., Pseudomonas aeruginosa and E.coli was chosen for further analysis. The gram +ve LAB grew well at pH 5 and 7, at temperature 25 and 37◦C, 5% Nacl solution and no growth was observed at pH 4 and 9, at temperature 4 and 45◦C, 15 and 25 % Nacl concentration. The LAB was catalase, gelatin and oxidase –ve and non acid fast. Further, through 16s rRNA gene sequencing it was identified as Lactococcus garvieae The study showed that the Lactococcus garvieae produce bacteriocin that inhibits a wide variety of pathogenic microbes which suggested that it can be used as an alternative type of antibiotic.

Keywords: Lactococcus garvieae, Bacteriocin, Antimicrobial susceptibility, Milk.

INTRODUCTION

(The organism's names should be italic and after Lactococcus garvieae full point should come)

This study was conducted to isolate lactic acid bacteria (LAB) from cow's milk and characterize through morphological, physiological, biochemical, carbohydrate fermentation and its antimicrobial property. The bacteria were isolated by serial dilution of milk samples and confirmed through biochemical, physiological and 16s rRNA sequencing. The antibiotic susceptibility test of bacteria, antimicrobial test of bacteriocin by agar well diffusion assay was carried out. A total of 12 LAB has been isolated among them one isolate which showed good antimicrobial activity against Gram-positive bacteria viz., Staphylococcus aureus, Bacillus subtilis and Bacillus cereus and gram-negative bacteria viz., Pseudomonas aeruginosa and E.coli was chosen for further analysis. The gram +ve LAB grew well at pH 5 and 7, at temperature 25 and 37◦C, 5% Nacl solution and no growth was observed at pH 4 and 9, at temperature 4 and 45◦C, 15 and 25 % Nacl concentration. The LAB was catalase, gelatin and oxidase –ve and non acid fast. Further, through 16s rRNA gene sequencing it was identified as Lactococcus garvieae. The study showed that the Lactococcus garvieae produce bacteriocin that inhibits a wide variety of pathogenic microbes which suggested that it can be used as an alternative type of antibiotic.

MATERIALS AND METHODS

Cultures

The indicator organisms namely *Bacillus cereus, Staphylococcus aureus, Bacillus subtillus E.coli,* and *Pseudomonas aeruginosa* were taken for the inhibitory activity which were isolated from Bovine mastitis infected udder milk and maintained in the Department of Biotechnology and Microbiology, Karnatak University, Dharwad.

Isolation of LAB

Raw unpasteurized milk samples of Cow were collected from the local Dairy (Vinay Dairy) in Dharwad, Karnataka, India. During the lactation period under aseptic conditions in sterile tarsons tubes and brought to the lab, processed within one hour and utilized for further studies. The milk sample was serially diluted in distilled water and plated on Man Rogosa Sharpe Agar and incubated at 37^{0}C for 48-72 hrs. The colonies with typical characteristics namely pure white, small of 2-3mm diameter with entire margins were hand picked from each petri plate. The cultures were identified according to their morphological, physiological, biochemical and molecular characteristics up to the genetic level [Harrigan et al., 1970**;** Sneath et al 1986; Holzapfel et al., 1991].

Biochemical Characterization

Bacteriocin producing strains were gram stained and examined microscopically for cellular morphology and gram-stain phenotype. Catalase activity was tested by spotting colonies with 3% hydrogen peroxide. Growth was assayed in MRS broth at 4, 25, 37 and at 45 oC as well as at pH of 4, 5, 7, 9 and incubated at 37 oC. Salt tolerance was tested with 5, 15 and 25 % (w/v) NaCl in MRS broth. The production of acid and CO2 from glucose was tested in MRS broth containing Durhams tube with citrate omitted [Schillinger et al., 1987]. An assay for gelatin hydrolysis was performed in accordance with [Harrigan et al 1970]. Ability to ferment various carbohydrates was evaluated using MRS broth [Schillinger et al., 1987].

Isolation of genomic DNA

Extraction of DNA from culture broth (~10-20mg). The first step is to add Lysis solution containing SDS to broth and Proteinase K. The lysate is then incubated at high temperature to digest proteins and release nucleic acid, followed by removal of proteins and cellular debris by precipitation and centrifugation. The nucleic acid is then recovered from the clarified lysate by isopropanol precipitation. Yields are dependent on the type and amount of sample, but are typically between 10-100 µg per prep.The purity of the nucleic acid as determined by UV absorbance ratio (Abs 260/Abs 280) is typically between1.8-2.0.

Identification of bacteria by sequencing of the 16S rRNA gene

The genomic DNA was isolated from the isolate. Amplification of the 16s rRNA gene was performed using the universal primers. Sequence analysis was carried out using NCBI online tools.

16S BACTERIAL PRIMER SEQUENCE

63F-5' CAG GCC TAA CAC ATG CAA GTC 3'
1387R-5' GGG CGG AGT GTA CAA GGC 3'

Maintenance of microorganisms

All the lactic acid bacterial cultures were maintained at 4°C in MRS broth. Pathogenic microorganisms were maintained at 4°C in Brain Heart Infusion broth. All the bacterial cultures were sub-cultured at 10 days interval.

Production of bacteriocin like inhibitory substances (BLIS)

Isolated bacterial cultures were inoculated in 100 ml of MRS broth and incubated at 37°C for 24 h. The broth was subjected to centrifugation at 12,000 rpm for 20 mins, the resulting cell residue was discarded giving rise to a cell free supernatant (CFS). The pH of supernatant was adjusted to 5.0 with 1N NaOH and then subjected for rotary flash evaporator, resulting solution thus obtained has been designated as BLIS. For inhibitory activity BLIS was filter sterilized by 0.22µm membrane filter paper (Millipore, India) to carry out the anti-microbial activity by well diffusion assay [VijVijai Pal et al., 2005].

Antimicrobial susceptibility testing

Agar Well Diffusion Assay (AWDA)

100µl of the 24 h old test cultures was inoculated onto the nutrient agar plates by the spread plate method. 3 wells of diameter 6mm were made in each of the plates. These wells were filled with 15µl, 30µl and 45µl of BLIS and the plates were incubated at 37° for 24hrs [Schillinger et al., 1987]. The inhibition zone was measured in millimeter using zone interpretation scale (HiMedia, Mumbai).

Antibiotic susceptibility test

Colonies of *Lactococcus garvieae* were inoculated in MRS broth at 37 °C for 24 h. A sterile spreader dipped into the bacterial suspension was spread evenly on the surface of the MRS agar plate. The inoculated plate was allowed to dry before placing the diffusion discs containing antibiotics. The antibiotics used for the test are Ceftriaxone (30mcg), Penicillin (10mcg), methicillin (5mcg), Cephalothin (30mcg), cloxacillin (30mcg), Tetracycline (30mcg), Ampicillin (10mcg), Amoxyclav (30mcg), Erythromycin (15mcg), Rifampicin (30mcg), Ciprofloxacin (5mcg), Cefpodoxime (10mcg), Carbenillicin (100mcg), Gentamicin (10mcg), which are procured from Hi-media Laboratories Pvt, Ltd Mumbai. The zone of inhibition is measured by a zone interpretation scale from Hi-media.

RESULTS

Physiological and Biochemical Characteristics

The isolated colony weakly grows at temperature of 4°c and a pH of 4 and 9, however it grown profusely at temperature 37° c and pH of 5 and 7. The organism grown well in 5% Nacl concentration and weak in 15% and shows no growth in 25% of Nacl concentration. The isolated colony is gram positive, gelatin negative, catalase negative, oxidase negative, and non-acid fast. It is non-motile and non sporulating.

Table 1: Physiological and Biochemical characteristics of *Lactococcus garvieae* strain

Tests	*Lactococcus garvieae*
Morphology	Cocci
growth at temp (◦C)	
4	W+
25	+
37	++
45	-
growth at pH	
4	W+
5	+
7	++
9	W+
Nacl (%)	
15	W+
25	-
Motility	Non-motile
Spore	Non sporulating
catalase	-
oxidase	-
gelatine	-
acid fast	-

Luxurious growth (++), Growth (+), Weak growth (W+), No growth (-)

Carbohydrate Utilization Test

The results have shown that the isolated colonies fermented sucrose, glucose, maltose, galactose, lactose, fructose, mannose and it weakly fermented xylose and does not ferment mannitol. Hence, it shows that it may belong to *Lactococcus species*.

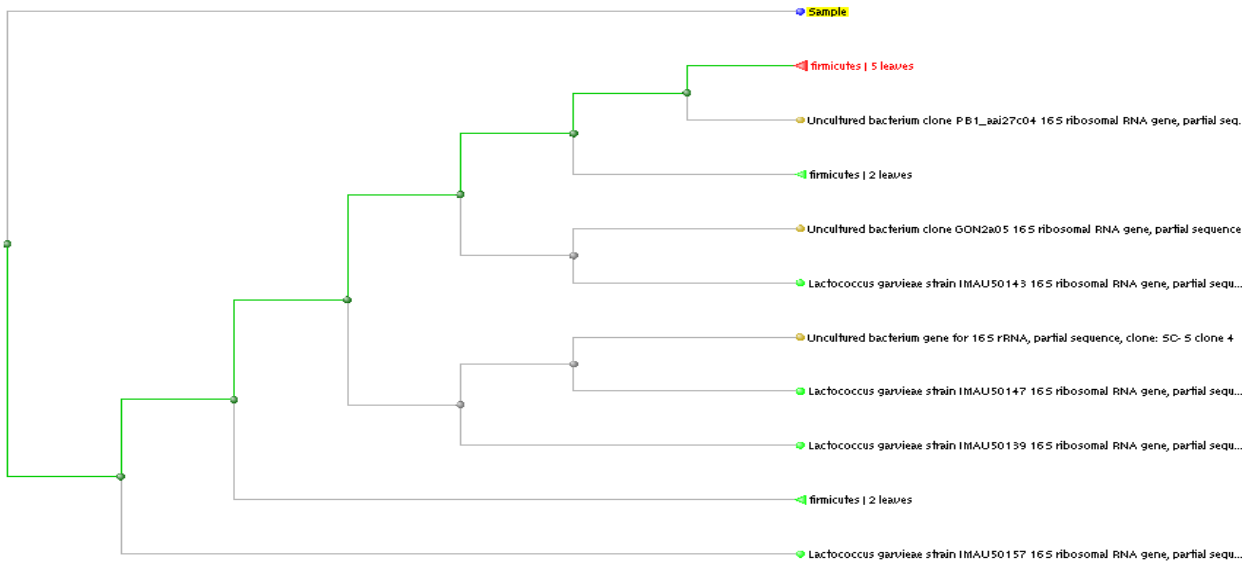
Table 2: Carbohydrate utilization test.

Carbohydrate	*Lactococcus garvieae*
Sucrose	+
Lactose	+
Fructose	+
Glucose	+
Galactose	+
Xylose	+*
Mannitol	-
Mannose	+

Test Legend: growth (+), no growth (–), delayed growth (+*)

The evolutionary history was inferred using the NCBI distance tree method. The evolutionary distances were computed using the p distance method and are in the units of the number of base differences per site. The analysis involved 11 nucleotide sequences. Fig 1 showing the PCR amplification of 16sRNA gene of Lactococcus garveie in comparison to 1kb ladder sequence.

Sequencing of the16S rRNA gene



LANE 1 LANE 2

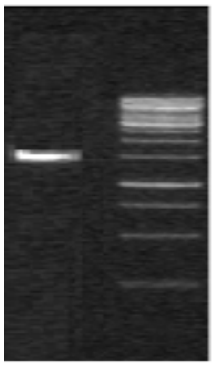


Fig 1: PCR amplified product in Agarose gel (Lane 1, PCR amplified sample; Lane 2, fermentas 1kb ladder)

Antimicrobial susceptibility testing

Agar Well Diffusion Assay (AWDA)

Table 3: Inhibitory activity of *Lactococcus garvieae* against various indicator bacteria.

Indicator Strains	*Lactococcus garvieae* (Diameter of Inhibition Zone in mm)		
	15 µl	30 µl	45 µl
Escherichia coli	19 mm	22 mm	30 mm
Bacillus subtilis	___	26 mm	31 mm
Bacillus cereus	16 mm	21 mm	24 mm
Staphylococcus aureus	21 mm	25 mm	27 mm
Pseudomonas aeruginosa	17 mm	___	21 mm

Zone size in mm. 6mm well diameter

The present results revealed that the bacteriocin produced from LAB showed moderate inhibition zone. At different concentrations of bacteriocin such as 15µl, 30 µl and 45µl showing zone of inhibition of 21 mm, 25 mm and 27 mm respectively against *Staphylococcus aureus*. Further, it showed 0 mm, 26 mm, 31 mm of zone of inhibition at concentration of 15 µl, 30 µl and 45 µl respectively against *Bacillus subtilis* and at concentration of 15 µl, 30 µl and 45 µl the bacteriocin showed zone of inhibition of 16 mm, 21 mm, 24 mm against *Bacillus cereus* and showed moderate inhibitory zone at 15µl and 30 µl and maximum at 45µl in case of gram negative organism like *E. coli* showing zone of inhibition of 19 mm, 22 mm, 30 mm respectively and the bacteriocin showed 17 mm, 21 mm zone of inhibition at concentration of 15 µl and 45 µl against *P. aeruginosa*.

Zone size in mm. 6mm well diameter

Antibiotic susceptibility test

The antibiotic susceptibility test showed that Lactococcus garvieae was sensitive to erythromycin, ampicillin, streptomycin and gentamycin but was resistant to penicillin, methicillin, cefpodexime, cephalothin and succeptible to carbenicillin, Rifampicin, Amikacin, Tetracycline, Ciprofloxacin, Cefriaxone, Cloxacillin, and Norfloxacin.

Table 4: Antibiotic susceptibility test against *Lactococcus garvieae.*

Antibiotic	Zone size in mm
Carbenicillin	+
Erythromycin	++
Gentamycin	++
Cefpodexime	+++
Penicillin	+++
Rifampicin	+
Amikacin	+
Ampicillin	++
Tetracycline	+
Ciprofloxacin	+
Cefriaxone	+
Cloxacillin	+
Norfloxacin	+
Methicillin	+++
Streptomycin	++
Cephalothin	+++

Sucseptible (+), Sensitive (++), Resistant (+++)

DISCUSSION

On the basis of morphological, physiological and biochemical characters, sugar utilization pattern and through 16S rRNA gene sequencing this strain was identified as *Lactococcus garvieae*, which was Gram-positive cocci did not produce CO_2 from glucose, and it is catalase, oxidase, gelatin negative and non acid fast. These results are in accordance with *Pediococcus* bacteria as reported earlier [Garvie et al., 1986; Facklam et al., 1995]. Luxuriant growth was observed for *Lactococcus garvieae* at 37 °C while it was weak at 10 °C

and did not could grow at 45 °C. Molecular identification based on 16S rRNA gene sequence analysis the LAB isolate showed 99% similarity with *Lactococcus garvieae*. These results are in similar to *P. acidilactici* strain [Mahantesh et al., 2010]**.** In the present study the bacteriocin like inhibitory substances of *Lactococcus garvieae* showed the highest inhibition at concentration of 45µl against gram-positive bacteria namely *B. cereus S. aureus* and *Bacillus subtillus.* The gram-negative organisms namely, *P. aeruginosa* and *E.coli,* showed less inhibition zone compared to gram-positive organisms, which showed that the bacteriocins of lactic acid bacteria were more active towards gram-positive organisms compared to gram**-**negative organisms [Jack et al., 1995]**.** The reason for the observed activity may be due to the gram negative organisms that have an outer protective membrane, which covers the cytoplasmic membrane and peptidoglycan layer [Boziaris et al., 1999].

The antibiotics are used to test the susceptibility of *Lactococcus garvieae*; it was sensitive to erythromycin, ampicillin, streptomycin and gentamycin but was resistant to penicillin, methicillin, cefpodexime and cephalothin [Diler et al., 2002]. The antibiotics are most often used to control *Lactococcosis* rainbow outbreaks in turkey. The susceptibility of these LAB isolates tested against 16 different types of antibiotics in the present study showed that they are susceptible to the β-lactam group of antibiotics (penicillin G and ampicillin) and other principal types of antibiotics which include erythromycin, methicillin, gentamycin and tetracycline. β-lactam which is bactericidal became the most extensively used beneficial class of antibiotic because of their broad antibacterial spectrum and excellent safety measure. They stop the bacterial cell wall synthesis and lethal against gram positive bacteria. Erythromycins belong to macrolides group and have a similar range of action and efficacy similar to that of penicillin. These groups of antibiotics bind to ribosome, blocks protein synthesis (bacteriostatic) and are effective against gram-positive microorganisms [Liasi et al., 2009], selectively accumulated compared with gram negatives), the bacteriocin may be used as an alternative type of antibiotic [Kaur et al., 2012]. In the present study carbohydrate utilization pattern of *Lactococcus garvieae* revealed that it has the ability to ferment maltose, glucose, lactose, fructose, sucrose, mannose, and galactose but could not ferment mannitol and arabinose and showed delayed fermentation of xylose. In case of lactose the results are similar to that of *L. garvieae* strains from dairy origin are all lactose fermenters **[**Teixeira et al., 1996; Fortina et al., 2007]. Ability to ferment maltose is a special character of *Lactococcuus garvieie* while *P. acidilactici* could not ferment it [Garvie et al., 1986; Facklam et al., 1995; Barros et al., 2001] due to the absence of specific enzymes. On the basis of all these properties it is found to be homofermentative and did not produce CO_2 from glucose.

CONCLUSION

The present study indicated that *Lactococcus garvieae* isolated from cow's milk is effective in inhibiting the pathogenic microorganisms and will act as obstruction by developing its antimicrobial actions in the host system of defense. The inhibitory spectrum of the bacteriocin like antimicrobial substance has a potential role as an alternative form of antibiotic. The possible resistance mechanism of bacterioicn secreted by *Lactococcus garvieae* against a wide variety of bovine mastitis pathogens may enable to the development of alternative types of antibiotics.

ACKNOWLEDGEMENT

The authors are thankful to the Department of Biotechnology New Delhi, for providing Bioinformatics Infrastructure lab facility, DBT-KUD-IPLS program (BT/PR14555/INF/ 22/126/ 2010), DBT, New Delhi and Postgraduate Department of Studies in Biotechnology and Microbiology, Karnatak University, Dharwad for providing all the necessary facilities.

REFERENCES

1. Vanden-Berg, DJC, Smits A, Pot B, Ledeboer AM, Kersters K, Verbakel JMA, Verrips CT. Food Biotechnol 1993; 7(3): 189-205.
2. Furutan NP, Breiman RF, Fischer MA, Facklam RF. *Lactococcus garvieae* infection in humans: a cause of prosthetic valve endocarditis C297. Proceedings of the 91st America Society of Microbiology Conference. Dallas7 Am. Soc. Microbiol 1991; 109.
3. Fihman V, Raskine L, Barrou Z, Kiffel C, Riahi J, Bercot B, Sanson-Le Pors MJ. *Lactococcus garvieae* endocarditis, identification by 16S rRNA and sodA sequence analysis. J. Infect 2005; 52: e3-e6.
4. Teixeira, Ml, Merquior, VN, Vianni C, Carvaho MCE, Fracalanzza MGS, Steigerwalt Sel, Brenner AG, Facklam DJ. Phenotypic and genotypic Characterization of atypical *Lactococcus garvieae* strains isolated from water buffalos with subclinical mastitis and confirmation of L. garvieae as a senior subjective synonym of Enterococcus seriolicida. International Journal of Systematic Bacteriology 1996; 46: 664- 668.
5. Cleveland, J, Montville TJ, Nes IF, Chikindas ML. Bacteriocins: Safe, natural antimicrobials for food preservation. Int. J. Food Microbiol, 2001; 71: 1–20.
6. Juan Borrero, Dag A. Brede, Morten Skaugen, Dzung B Diep, Carmen Herranz, Ingolf F. Nes, Luis M. Cintas and Pablo E. Hernández. Characterization of Garvicin M. L, a Novel Circular Bacteriocin Produced by *Lactococcus garvieae* DCC43, Isolated from Mallard Ducks (*Anas platyrhynchos*) Appl. Environ. Microbiol. DOI 10.1128/AEM.01173-10, 2011; 77(1): 369-373.
7. Harrigan WF, and Margaret E, McCance. Laboratory methods in food and dairy microbiology, Academic Press Inc., 1970; 224.
8. Sneath, PHA, Mair, NS, Sharpe, ME, Holt JG. Bergey's Manual of Systematic Bacteriology, Vol. 2, Williams and Wilkins, Baltimore 1986; 1208-1234.
9. Holzapfel WH, and Schillinger U. The genus Lcu- 'o~losro. In: Balows A, Triiper HG, Dworkin M, Harder W, and Schleifer KH. (editors). The Prokaryotes, Vol. 11. 2nd edition. Springer-Verlag. New York. 1991; 1508-1534.
10. Schillinger, U, Lucke FK. Food Microbial, 1987; 4: 99-208.
11. VijVijai Pal, Jamuna M, and Jeevarathnam K. Isolation and characterization of bacteriocin producing lactic acid bacteria from a south indian special dosa (Appam) batter. J. Culture Collection, 2005; 4: 53-60.
12. Garvie, EI, Sneath PH, Mair NS, Sharpe ME, Holt JG. (Eds), Genus Pediococcus. In: Bergey's manual of systematic bacteriology Vol. 2, Baltimore: The Williams & Wilkins Co., 1986; 1075-1079.
13. Facklam RR, Elliot JA. Identification, classification, and clinical relevance of catalase- Negative, Gram-positive cocci, excluding the streptococci and enterococci. Clin Microbiol. Rev.1995; 8: 479-495.
14. Cai Y, Kumai S, Owaga M, Benno Y, Nakase T. Appl. Environ. Microbiol 1999; 65(7): 2901- 2906.
15. Mahantesh P, Ajay Pal T, Anand and Ramana KV. Isolation and characterization of lactic acid bacteria from curd and cucumber Indian J of Biotechnology, April, 2010.
16. Jack, RW, Tagg, JR. and Bibek Ray. Bacteriocins of gram-positive bacteria. Microbiol Reviews, 1995; 59(2): 171-200.
17. Boziaris, IS, and Adams, NM. Effect of chelators and nisin produced in situ on inhibition and inactivation of gram negatives. Int. J. Food Microbiol 1999; 53: 105-113.
18. Diler O, Altun S, Adiloglu AK, Kubilay A, Isikli B. First occurrence of Streptococcosis affecting farmed rainbow trout (Oncorhynchus mykiss) in Turkey. Bull. Eur. Ass Fish Pathol 2002; 22: 21-25.
19. Liasi, SA, Azmi, TI, Hassan, MD, Shuhaimi, M, Rosfarizan, M and Ariff AB antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu Malaysian Journal of Microbiology, 2009; Vol 5(1): 33-37.
20. Kaur B, Garg N, Sachdev A. Optimization of bacteriocin production in pediococcus acidilactici ba 28 using response surface methodolog. Asian J Pharm Clin Res, Vol 6, Suppl 1, 2013, 192-195

21. Fortina, MG, Ricci G, Foschino R., Picozzi C, Dolci P, Zeppa G, Cocolin L, Manachini PL, Phenotypic typing, technological properties and safety aspects of *Lactococcus garvieae* strains from dairy environments. Journal of Applied Microbiology 2007; 103: 445-453.
22. Barros RR, Carvalho GS, Peralata JM, Facklam RR, Teixeira LMJ. Clin Microbiol 2001; 39 (4): 1241-1246.