Analysis of Nucleic Acids in *Malassezia furfur* Serovars A, B and C

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Background **: Recently *Malassezia (M.) furfur* fungemia has been increasingly recognized in premature infants and adults receiving parenteral nutrition. Accordingly, analysis of nucleic acids in M. *furfur* serovars and strain typing methods based on genetic differences and similarities are required for epidemiological studies.**

Objective **: This study was done to analyze nucleic acids in M. *furfur* serovars A, B and C and to adapt the method of restriction fragment length polymorphism (RFLP) analysis of DNA to differentiate the strains of M. *furfur* serovars for use in epidemiological studies.**

Methods **: Cellular nucleic acids were extracted from the strains of M. *furfur* serovars and electrophoresed, followed by digestion of DNA and electrophoresis of the resultant DNA fragmegments.**

Results **: Each of the six strains, grown both on solid medium and liquid medium, revealed a genomic DNA. Interestingly, unique extra bands of RNA were observed in four of the six strains which had grown on solid medium. These bands were also seen in three of them grown in broth. The size of these bands were from 0.5 to 5.0 kbp by comparison with a '1 kb DNA ladder'. The restriction patterns generated by EcoR I, Hae III, Hind III, and Hinf I were not unsuccessful. The DNA from serovar B was insensitive to the above restriction enzymes**

Conclusions **: Although DNA was extracted from the strains, the amounts were not thought to be enough for RFLP analysis and the DNA from the serovar B was insensitive to the above restriction enzymes. Thus, further development of an extraction method of DNA is required for obtaining enough DNA from M. *furfur* serovars, and other restriction enzymes would have to be investigated for their ability to differentiate strains of M. *furfur* in epidemiological studies. Also, further investigation of RNA bands might be able to adapt them for a typing method. (Ann Dermatol 9:(1)1~7, 1997).**

Key Words : *Malassezia furfur* serovars A, B and C, RFLP, Nucleic acids

M. *furfur* is a lipophilic yeast which is not only a member of the normal flora on human skin predominantly in regions of high sebum excretion rate but is also associated with several cutaneous diseases such as pityriasis versicolor, pityrosporum folliculitis, and seborrheic dermatitis[1,2]. Recently M. *furfur* has increasingly been recognized as a cause of serious systemic infection in premature neonates and adults receiving parenteral lipid nutrition through indwelling vascular catheters[3]. Administration of fat emulsions appears to favour the growth of M. *furfur*, leading to colonization of the catheter and subsequent infection[3]. The source of M. *furfur* causing catheter sepsis in infants may be due to contamination of the catheters by nurses or clinicians although it has been suggested that contaminated fat emulsions should be sought as a possible

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source of infection[4,5].

Cultures of M. *furfur* showed considerable variation in cell shape, colonial morphology and antigens according to the medium used. Midgley[6,7] divided M. *furfur* isolates into *Pityrosporum(P.) orbiculare* and three forms of *P. ovale* using a medium adapted from that devised by Dixon[8]. Using a medium with whole-fat cow,s milk formulated by Leeming and Notman[9], Cunningham et al[10] classified M. *furfur* isolates into three morphological groups on the basis of cultural characteristics and three serovars A, B and C based on serological differences in cell surface antigens. These morphological and serological classifications revealed a remarkable degree of correspondence. These morphological and serological differences in M. *furfur* variants are, however, insufficient for detailed epidemiological comparisons. Therefore, the most valuable method to classify and differentiate strains of M. *furfur* is one based on genetic differences or similarities. The aim of the present study was to analyze nucleic acids in M. *furfur* serovars A, B and C and to adapt the method of restriction fragment length polymorphism (RFLP) analysis of DNA to differentiate the strains of M. *furfur* serovars for use in epidemiological studies.

MATERIAL AND METHODS

Malassezia Strains

In the present study two strains of serovar A (24CC FL, 21HK CHL), two strains of serovar B (31ML CHS, 34CJ CHS) and two strains of serovar C (35JR FS, 19BD CHS) were analyzed. These strains were isolated from healthy human skin from volunteers working in the department of Microbiology, University of Leeds.

Growth on Solid Medium and Storage of Cells

Each strain of M. *furfur* serovar A was inoculated onto 5 plates of Leeming and Notman agar (1% w/v peptone (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England), 0.5% w/v glucose (Sigma Chemical Co., St. Louis, MO, U.S.A.), 0.01% w/v yeast extract (Oxoid), 0.4% w/v desiccated ox bile (Oxoid), 0.1% v/v glycerol (Sigma), 0.05% w/v glycerol monostearate (BDH, Merck Ltd.,Poole, BH15 1TD, England), 0.05% v/v Tween 60 (Sigma), 1% v/v whole fat cow's milk (Associated Dairies, Leeds, UK), 1.2% w/v agar (Oxoid), chloramphenicol (Sigma) 50 mg l^{-1}, cycloheximide (Sigma) 200 mg l^{-1}) and incubated for 3 days at 34℃. Each strain of M. *furfur* serovars B and C was inoculated onto 10 plates of Leeming and Notman agar and incubated for 5 days at 34℃. The cells were then removed from the agar and resuspended in 1 M sorbitol (Sigma) at a concentration of 2×10^9 ml^{-1} and frozen at 20℃ until required.

Growth in Liquid Medium and Storage of Cells

A heavy inoculum of each strain of M. *furfur* to be studied was transferred to 10 ml of liquid medium (1% w/v peptone (Oxoid), 1% w/v glucose (Sigma), 0.2% w/v sodium taurocholate (BDH), 0.01% w/v yeast extract (Oxoid), 0.1% v/v glycerol (Sigma), 0.05% v/v Tween 60 (Sigma), 0.04% v/v whole fat cow's milk (Associated Dairies, Leeds, UK), chloramphenicol (Sigma) 50 mg l^{-1}, cycloheximide (Sigma) 200 mg l^{-1}) and incubated at 34C with shaking. When turbid, the cultures were transferred to 20 ml of liquid medium and incubated in the same way. After 24 hours incubation, this culture was used to inoculate 100 ml of liquid medium which was incubated in the same way. When the cultures reached the mid-exponential phase after about 22-26 hours the yeast cells were harvested by centrifugation (2.5×10^3 r.p.m. for 20 mins). The cells were then washed in 1 M sorbitol (18.22 g sorbitol (Sigma), distilled water to 100 ml), centrifuged at 1.3×10^4 r.p.m. for 5 mins and finally resuspended in 1 M sorbitol at a concentration of 2×10^9 ml^{-1} and frozen at −20℃ until required.

Washing and Lysis of Cells

Aliquots of cells were thawed, centrifuged at 1.3×10^4 r.p.m. for 5 mins, and the sorbitol was poured off. The cells were then treated with acetone (BDH) and ethanol (Hayman Ltd.Witham, Essex, CM8 3YE, England) to remove lipid from the cell wall and weaken the outer layer, by adding 400μl acetone, 400μl ethanol and 200μl 0.01 M EDTA (0.208 g EDTA (Sigma) plus 25 ml distilled water). The EDTA was added to prevent the action of nucleases. The cells were then left on ice for 15 mins, after which they were centrifuged at 1.3×10^4 r.p.m. for 5 mins. The cells of serovars A and C to be treated with Zymolyase-20T (ICN Biomedicals Ltd., High Wycombe, Bucks, HP13 7DL, U.K.) were then

washed three times in Zymolyase buffer* whilst those of serovar B to be treated with Novozym-234 (Novabiochem Corp., La Jolla, CA, U.S.A.) were then washed three times in Novozym buffer (citrate-phosphate buffer was prepared by adding 42 ml of 0.2 M citrate (Sigma) and 58 ml of 0.4 M phosphate (BDH). The pH of this was then adjusted to 5.6 by addind small amounts of 0.2 M citrate and 0.4 M phosphate. Then, 50 ml of citrate-phosphate buffer, pH 5.6 was mixed with 10.405 g of EDTA and 21.846 g of sorbitol and the volume made up to 100 ml with distilled water. After autoclaving, 0.1 ml of 2-mercaptoethanol was added to form the final Novozym buffer. Zymolyase-20T was used at a final concentration of

20U. ml^{-1} and Novozym-234 at 2.5 mg ml^{-1}. The cells in Zymolyase-20T and to form the final Novozym buffer. Zymolyase-20T was used at a final concentration of 20 U. ml^{-1} and Novozym-234 at 2.5 mg ml^{-1}. The cells in Zymolyase-20T and Novozym-234 were incubated at 37℃ for 18 hours and at 30℃ for 38 hours, respectively. After incubation of the cells with the appropriate enzyme, 10% sodium dodecyl sulphate (Sigma) solution was added to give a final concentration of 0.4% to lyse the cells and release the DNA. and the cells were heated tō 70℃ for 30-35mins.This lyzed the cells and released the nucleic acids.

Extraction and Electrophoresis of Nucleic acids

To extract the DNA from the cells one volume of cells and one volume of Tris (Sigma)-saturated phenol (Fisons Instrument, Altrincham, Cheshire, WA14 5TP, U.K.) were mixed, vortexed and centrifuged at 1.3×10^4 r.p.m. for 2 mins. The upper yellow phenol layer was discarded and one volume of chloroform was added to the lower aqueous layer, vortexed and centrifuged at 1.3×10^4 r.p.m. for 2 mins. The upper aqueous layer, which was separated from the lower chloroform layer by a white precipitate of protein, was removed to a clean tube and an equal volume of chloroform added, vortexed and centrifuged at 1.3×10^4 r.p.m. for 2 mins. The aqueous layer was again removed and 1/10 volume of 3 M sodium acetate (24.6 g of sodium acetate (BDH), distilled water to 100 ml) and 2.5 volume of absolute ethanol held at -20℃, added. The tube was centrifuged at 1.3×10^4 r.p.m. for 5 mins. The pellet formed consisted of DNA and was washed in 70% w/v ethanol held at -20℃ and centrifuged at 1.3×10^4 r.p.m. for 5 mins. The ethanol was decanted and the pellet allowed to dry. Finally, 30μl of TE buffer (1 ml of 1 M Tris-HCl, pH 8.0, 0.2 ml of 0.5 M EDTA, pH 8.0, distilled water to 100 ml) was added and the nucleic acids allowed to dissolve overnight.

Electrophoresis of the nucleic acids was carried out in 0.8% agarose which was composed of 0.64 g agarose (Sigma), 8 ml of TBE {10×} and 72 ml distilled water. 10x TBE solution was prepared by adding 108 g Tris, 55 g boric acid (Sigma) to 40 ml 0.5 M EDTA, pH 8.0 and the volume made up to 1000 ml with distilled water. A gel tray was cleaned with alcohol and the end sealed with autoclave tape. A comb was secured in place and the agarose poured into the gel tray and allowed to set at room temperature. The comb and tape were then removed and the gel tray placed in the wide Mini-Sub cell (Bio-Rad Lab., Richmond, CA, U.S.A.) containing 1×TBE with the gel submerged to a depth of 1-2 mm. The samples were then loaded into the gel. From each sample, 5μl of the DNA sample was mixed with 1μl of the sample buffer and 5μl of sterile distilled water and placed into the appropriate well in the gel. Additionally, a 5μl aliquot from each sample was incubated at 37℃ for 30 mins with 1.25μl of RNase A(10 mg l^{-1}) (Sigma) and mixed with 1μl of 10x loading buffer (30% Ficoll (Sigma), 0.25% bromophenol blue (BDH), and 0.2 M EDTA, pH 8.0, in 10×TBE) and placed into the appropriate well in the gel. The lid of the wide Mini-Sub cell was replaced and connected to a power supply (LKB Wallac 2197, LKB Instrument, Inc., Bromma, Sweden). The sample was then electrophoresed at a constant voltage of 80 V towards the anode. To visualise the nucleic acids, gels were stained in a solution of ethidium bromide (2 g ml^{-1}) for 20 mins. 1 Kb DNA ladder GIBCO BRL, Life Technologies, Inc., Gaithers-

*To make 50 mM KH2PO4 [BDH], which formed the basis of the Zymolyase buffer, 50 ml of 0.1 M KH2PO4 and 39 ml of 0.1 M NaOH [Sigma] were mixed and the pH of the solution adjusted to 7.4 using small amounts of 10 M HCl [Sigma]. Then 50 ml of 50 mM KH2PO4, pH 7.4 was added to 18.22 g sorbitol and the volume adjusted to 100 ml with distilled water. After autoclaving, 0.1 ml of 2-mercaptoethanol [Sigma] was added to form the final Zymolyase buffer.

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burg, MD, U.S.A. was used as size references. They were then observed under ultraviolet light (Fotodyne Inc., Hartland, WI, U.S.A.) and photographed using a Polaroid CU-5 camera.

Restriction Endonuclease Digestion and Gel Electrophoresis

Restriction endonucleases (*Eco*R I, *Hae* III, *Hind* III, and *Hinf* I) were obtained from GIBCO BRL. A 20μl aliquot of nucleic acid sample was treated with 5μl of RNase A (10 mg ml^{-1}) and digested with 1.5μl of restriction endonuclease and 3μl of core buffer for 2 hours at 37℃. To ensure complete digestion, an additional 1.5μl of enzyme was added to the reaction mixture and further incubated for 2 hours at 37℃. Digestion was stopped by heating the mixture to 65℃. Electrophoresis was performed on a 0.8 % agarose gel at a constant voltage of 60 V towards the anode. The gel was stained with ethidium bromide and the DNA bands were visualized under ultraviolet light.

RESULTS

Extraction and Electrophoresis of Nucleic acids

Each of six strains, grown both on solid medium and in liquid medium, subjected to the nucleic acid extraction and electrophoresis methods described above, revealed an upper broad identifiable band, varying in intensity, representing genomic DNA (Fig. 1). Interestingly, unique extra bands were observed from serovar A (24CC FL), serovar B (31ML CHS, 34CJ CHS), and serovar C (35JR FS) which had been grown on solid medium. These bands were also seen from serovar B (31ML CHS, 34CJ CHS), and serovar C (35JR FS) grown in broth (Fig. 1). These extra bands were digested after the nucleic acid sample was treated with RNase A (Fig. 2). On the basis of the RNase A treatment, these extra bands were identified as RNA. The sizes of the RNA bands were measured by comparison with a '1 kb DNA ladder'. The RNA

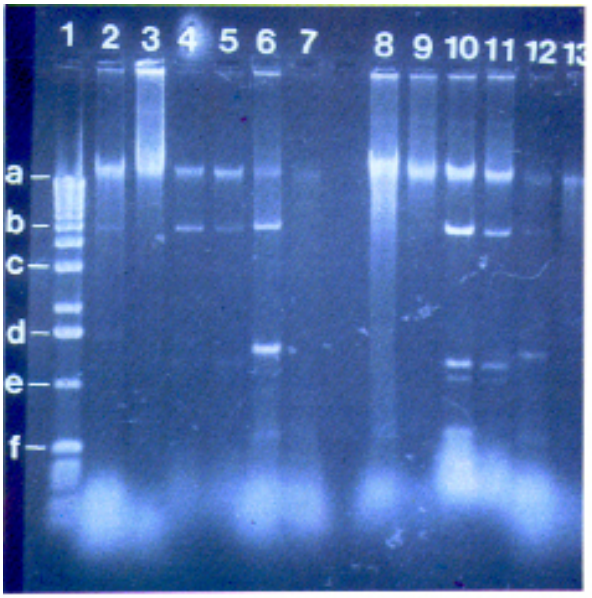

Fig. 1. Gel electrophoresis of DNA samples before RNase A treatment and digestion with restriction endonucleases. Six strains of M. *furfur* were grown on solid medium (lanes 2-7) and liquid medium (lanes 8-13). Lanes: 1 and 14 contain '1 kb DNA ladder'. Their sizes (in bp) are: (a), 12216; (b), 5090; (c), 3054; (d), 1636; (e), 1018, (f), 506, 517. 2 and 8, 24CC FL;3 and 9, 21HK CHL;4 and 10, 31ML CHS; 5 and 11, 34CJ CHS; 6 and 12, 35JR FS; 7 and 13, 19BD CHS. The first broad band is DNA, and the others are RNA.

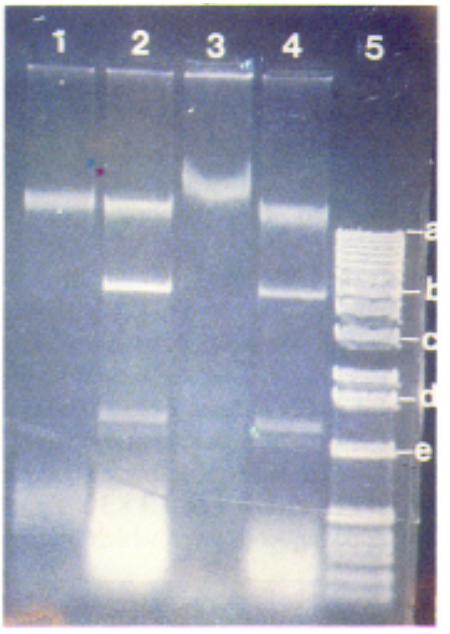

Fig. 2. Gel electrophoresis of DNA samples treated with RNase A revealed degradation of RNA bands. Lanes: 1, DNA sample of 31ML CHS treated with RNase A; 2, DNA sample of 31ML CHS was not treated with RNase A; 3, DNA sample of 34CJ CHS treated with RNase A; 4, DNA sample of 34CJ CHS was not treated with RNase A; 5 contains '1 kb DNA ladder'. Their sizes (in bp) are: (a), 12216; (b), 5090; (c), 3054; (d), 1636; (e), 1018. These two strains of serovar B were grown in broth.

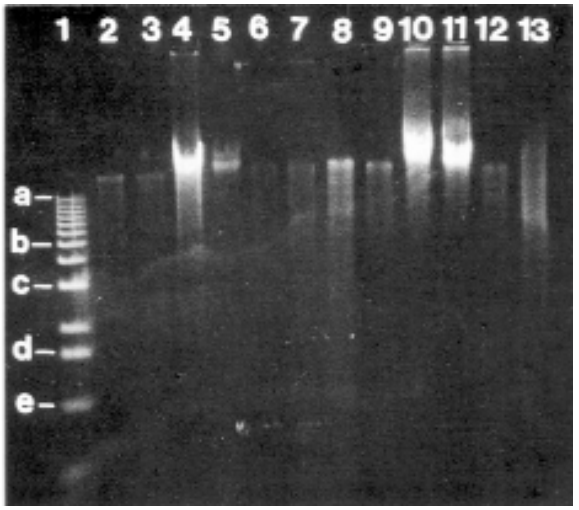

Fig. 3. Gel electrophoresis of DNA samples after RNase A treatment and digestion with restriction endonuclease EcoR I. Six strains of M. *furfur* were grown on solid medium (lanes 2-7) and liquid medium (lanes 8-13). Lanes: 1 contains '1 kb DNA ladder'. Their sizes (in bp) are: (a), 12216; (b), 5090; (c), 3054; (d), 1636; (e), 1018, (f), 506, 517. 2 and 8, 24CC FL;3 and 9, 21HK CHL;4 and 10, 31ML CHS; 5 and 11, 34CJ CHS; 6 and 12, 35JR FS; 7 and 13, 19BD CHS. The strain differentiation by RFLP with EcoR I was unsuccessful and yields of DNA were not thought to be enough for RFLP and DNAs from 31ML CHS and 34CJ CHS were insensitive to EcoR I.

band of 24CC FL is 5.0 kbp in size. 31ML CHS and 34CJ CHS on broth culture showed four and three RNA bands of 0.5-5.0 kbp in size, respectively, but these ones revealed one band of RNA when cultured on solid medium. From 35JR FS three RNA bands of 0.5-5.0 kbp in size were observed. 24CC FL, 31ML CHS and 34CJ CHS showed different banding patterns of RNA according to the media used.

Restriction Endonuclease Digestion and Electrophoresis

In order to carry out strain differentiation, the restriction patterns generated by EcoR I (Fig.3), Hae III, Hind III and Hinf I were examined.The RFLP analyses of DNA with the four restriction endonucleases were unsuccessful, and DNA of serovar B (31ML CHS, 34CJ CHS) was not digested with the four restriction endonucleases.

DISCUSSION

Although M. *furfur* constitutes part of the normal cutaneous flora, and it also causes minor cutaneous disorders, serious systemic infection due to this organism has been reported in premature infants and adults receiving parenteral fat emulsions[3,11]. Morphological and serological characteristics of M. *furfur* have been used to classify M. *furfur*[6,10,12,13]. However, these methods are not reliable for an epidemiologic tool and thus a genetic typing method is required for strain differentiation. Boekhout and Bosboom[14] studied karyotypes of the medically important yeasts M. *furfur*, M. *pachydermatis* and M.*sympodialis* by pulsed field electrophoresis. All the strains of M. *pachydermatis* revealed a similar karyotype. The karyotype of M.*sympodialis* was identical to part of strains currently classified as M. *furfur*. Their electrophoretic karyotyping of M. *furfur* did not discriminate between isolates. Van Belkum et al[11] performed genetic typing of *Malassezia* strains by PCR-mediated DNA fingerprinting using five PCR primers (ERIC IR, ERIC2, REP1R-I, REP2-I and BG-2). All isolates of M. *pachydermatis* displayed identical DNA banding patterns. The clinical isolates of M. *furfur* showed homogeneity in banding patterns except two strains which showed minor differences. They stated that their fingerprinting assays could not document genetic differences among the clinical isolates of either M. *furfur* or M. *pachydermatis*. fingerprinting was a *Malassezia* typing procedure that was to be preferred over the analysis of chromosomal polymorphisms by pulsed-field gel electrophoresis in this genus. Midgley and Schechtman[15] detected nine distinct patterns by restriction of total DNA with the enzyme Hae III (RFLP) in the fifty M. *pachydermatis* strains. al., 1990). In an attempt to adapt RFLP for use with M. *furfur*, the cell wall has to be lyzed. DNA isolation and purification from basidiomycetes are known to pose problems much more frequently than from endomycetous or euascomycetous fungi[16]. The ultrastructure of M. *furfur* revealed that both round and oval yeast cells possess a thick cell wall (0.12 l), layered with a regular series of indentations into the plasma membrane arranged in a spiral[17,18]. This thick cell wall is difficult to break. Van Belkum et al[11] extracted DNA from M. *pachydermatis* and M. *sympodialis* strains by treating cells with Novozym and developed another suitable procedure for the extraction of DNA from *Malassezia* species because

the cell wall of clinical M. *furfur* isolates appeared to resist enzymic digestion. Cell pellets were lyophilized for at least 60 hours. The resulting powder was resuspended in a buffer containing 100 mM EDTA, and 2% (v/v) Triton X-100, and the mixture was incubated at 37℃ for at least 2 hours. Thereafter, DNA was purified by affinity chromatography. However, in the present study DNA was extracted from the cells by enzymic digestion of the cell wall without using any mechanical breakage.

All strains investigated, irrespective of type of medium, showed a genomic DNA band. In addition to a genomic DNA band, a unique RNA band was seen from one strain of serovar A (24cc FL) on the solid medium but not in the broth. Two strains of serovar B (31ML CHS, 34CJ CHS) and one strain of serovar C (35JR FS) showed unique RNA bands regardless of type of medium. The solid and the liquid medium are a little different from each other qualitatively and quantitatively. The amounts of glucose and whole fat cow,s milk in the solid medium are not the same as those in liquid medium. The solid medium and the liquid medium do not contain sodium taurocholate and glycerol monostearate, respectively. These quantitative and qualitative differences in the formulae of two media may have an influence on the production of a RNA band from the strain of serovar A (24CC FL) and additional RNA bands from the two strains of serovar B in some way. However, it was also suggested that the extraction method had not always been effective or cells had lost the extra bands of RNA during the procedure. Although Anthony et al[19] mentioned a 6.4 kbp RNA 'plasmid' and a 1.8 kbp RNA 'plasmid' in M. *pachydermatis* strains, the RNA bands in the M. *furfur* serovars A, B and C had not been reported. An eukaryotic cell contains nuclear mRNA precursors, nuclear rRNA precursors, nuclear tRNA precursors and small nuclear RNAs, cytoplasmic ribosomal RNA, cytoplasmic transfer RNA, and cytoplasmic messenger RNA[20]. RNA bands of the M. *furfur* serovars were not thought to be any of the above mentioned RNAs. These appeared to represent a RNA virus which might have infected the cells of them. As a result, detailed investigation is required to elucidate the molecular characteristics of the RNA bands obtained from the M. *furfur* strains in this study. Many fungal viruses are frequently found in *Saccharomyces cerevisiae* and filamentous fungi[21]. Fungal properties in relation to viral infection are not defined in detail except for the killer-immune character in the yeasts and smuts and hypovirulence in the chestnut blight pathogen, *Cryphonectria parasitica*[22]. Families of RNA viruses infecting fungi include *Totiviridae*, *Partitiviridae*, *Hypoviridae*, and *Barnaviridae*[21]. The first three contain double-stranded RNA, and the other single-stranded RNA. The RNA size of 24CC FL grown on solid medium is 5.0 kbp. 31ML CHS and 34CJ CHS grown in broth showed four and three RNA bands (0.5-5 kbp in size), respectively, but the same strains grown on solid medium revealed one band of RNA. Three RNA bands (0.5-5 kbp in size) were observed from 35JR FS irrespective of type of media. The additional banding patterns of RNA from the serovars B and C on broth culture were different from each other. Considering the size of viral RNA and its host21, the RNA bands of serovars of M. *furfur* may belong to the family *Totiviridae*.

In this study RFLP analysis was carried out with EcoR I, Hae III, Hind III, and Hinf I. and assessed for its ability to differentiate the serovars and strains of the same serovars. However, the yield of DNA obtained from the serovars A, B and C was not thought to be large enough to allow RFLP analysis and and the above four enzymes could not digest the DNA of two strains of serovar B. Thus, further development of an extraction method is needed for obtaining enough DNA from M. *furfur* serovars, and other restriction enzymes would have to be investigated for their ability to differentiate strains of M. *furfur* in epidemiological studies. However, the presence of these RNA bands may in itself help to type and differentiate strains of M. *furfur* serovars A, B and C.

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