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First genotyping confirmation of *Pichia kudriavzevii* in subclinically mastitic cows in Iraq

Primeira confirmação de genotipagem de Pichia kudriavzevii em vacas com mastite subclínica no Iraque

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ABSTRACT

Fungal pathogens exist obviously in environment; therefore, animals may act as a source of infection to human. *Pichia kudriavzevii* is ubiquitous fungus of lastly great attention due to its potential use in biotechnology and processing of food, and controversial safety. This study aims to determining the prevalence rate of subclinical mastitis in lactating cows, and demonstration the presence of *Pichia kudriavzevii* in milk of positively mastitic cows using the molecular phylogeny. Totally, 400 adult lactating cows were subjected for collection an approximately 50 ml of fresh milk that tested initially with the California Mastitis Test (CMT); and then, positive samples have tested molecularly using conventional polymerase chain reaction (PCR). Some molecularly positive samples were analyzed phylogenetically for confirming of local isolates in the National Centre for Biotechnology Information (NCBI). Overall, 54.25% cases were positively reacted by CMT. According to score of positivity, 83.87%, 11.98% and 4.15% were showed weak, distinct, and strong positive infections, respectively. Targeting the ITS region, 28.11% of samples were reacted positively to *P. kudriavzevii* at 278 bp. Phylogenetic analysis of eight local *P. kudriavzevii* isolates showed the nucleotide alignment similarity and substitutions. Phylogenetic tree analysis revealed that the local *P. kudriavzevii* isolates were showed a genetic identity to the NCI-BLAST *P. kudriavzevii* Mexico isolates (KY646192.1) at total genetic changes ranged 0.0035-0.005%. In conclusion, this represents first molecular phylogenic study in Iraq implicates the presence of *P. kudriavzevii* in subclinical mastitic cows. Nationwide surveys are useful in monitoring udder health, studying the impact of structural changes, and estimating the factor(s) contribute in incidence of disease and the role of different fungi in it.

KEYWORDS: mycotic mastitis; bovine fungal infection; *Candida krusei;* conventional PCR; sequencing analysis.

RESUMO

Os patógenos fúngicos existem obviamente no meio ambiente; portanto, os animais podem atuar como fonte de infecção para os humanos. *Pichia kudriavzeviiis* é um fungo onipresente que merece grande atenção devido ao seu potencial uso em biotecnologia e processamento de alimentos, além de segurança controversa. Este estudo tem como objetivo determinar a taxa de incidência de mastite subclínica em vacas em lactação, e demonstrar a presença de *Pichia kudriavzevii* no leite de vacas positivamente mastíticas utilizando a filogenia molecular. No total, 400 vacas adultas em lactação foram submetidas à coleta de aproximadamente 50 ml de leite fresco que foi testado inicialmente com o teste de mastite Califórnia (CMT); e então, amostras positivas foram testadas molecularmente usando reação em cadeia da polimerase (PCR) convencional. Algumas amostras molecularmente positivas foram analisadas filogeneticamente para confirmação de isolados locais no Centro Nacional de Informações sobre Biotecnologia (NCBI). Um total de 54.25% dos casos foram reagidos positivamente pela CMT. De acordo com o escore de positividade, 83.87%, 11.98% e 4.15% apresentaram infecções positivas fracas, distintas e fortes, respectivamente. Visando a região ITS, 28,11% das amostras reagiram positivamente a *P. kudriavzevii* a 278 pb. A análise filogenética de oito isolados locais de *P. kudriavzevii* mostrou semelhança e substituições no alinhamento de nucleotídeos. A análise da árvore filogenética revelou que os isolados locais de *P. kudriavzevii* mostraram uma identidade genética com os isolados NCI-BLAST de *P. kudriavzevii* México (KY646192.1) com alterações genéticas totais variando de 0,0035-0,005%. Em conclusão, isto representa o primeiro estudo filogenético molecular no Iraque que confirmou a presença de *P. kudriavzevii* emleite de vacas com mastite subclínica. Os inquéritos a nível nacional são úteis no monitoramento da saúde do úbere, no estudo

do impacto das mudanças estruturais e na estimativa do(s) factor(es) que contribuem para a incidência da doença e o papel dos diferentes fungos.
PALAVRAS-CHAVE: mastite micótica; infecção fúngica bovina; Candida Krusei; PCR convencional; análise de sequenciamento.

INTRODUCTION

Worldwide, mastitis represents a frequent diseased condition of large reproductive and economic losses in dairy cattle (PÉREZ-MORALES et al. 2022). This condition characterizes by an inflammation process in the mammary tissue due to mainly intramammary infection or rarely by the physical and chemical etiologies (KUMAR et al. 2020). At field, intramammary infection has great importance as it recurrently occurred by a single and/or multiple microorganisms such as algae, bacteria, mycoplasma, viruses, and yeasts (MOREIRA et al. 2019, RIFATBEGOVIĆ et al. 2024). However, the classical mastitis pathogens may either contagious that survives within a host, or environmental that opportunistically invades the mammary glands (KIBEBEW 2017, HAIDER et al. 2023)

Based on presence or absence of clinical signs, this condition classifies as either clinical or subclinical, while following the duration of infection, its further divided into acute or chronic forms (COBIRKA et al. 2020). In clinical mastitis, abnormal signs on udder (redness, hotness, swelling, solidity and pain) and/or milk (watery or bloody milk and reduced milk production) in addition to systemic reactions (fever, depression, and loss of appetite) are the usual signs (CONSTABLE et al. 2016). Whilst, SCM is characterized apparently normal udder without obvious symptoms of inflammation on udder or milk, but there is under-detectable decreasing in milk yield with alterations in composition, pH and ion concentration of milk with obvious elevation in levels of somatic cells (UMAM et al. 2017, SALEEM et al. 2021).

In recent years, occurrence of fungal diseases has dramatic increases and severity in both humans and animals due to an increased number of outbreaks attributed to a novel species such as *Trichophyton* spp., *Aspergillus* spp. *Candida* spp., *Geotrichum*, and *Pichia* spp. etc. (CARPOURON et al. 2022, PAL 2023). *Pichia kudriavzevii*, formerly known *Candida krusei*, is an emerging, non-conventional budding yeast belongs to the Pichiaceae Family under the Saccharomycetales Order of Fungi Kingdom, which discovered firstly in 1839 in a typhus patient (YADAV et al. 2012, JAMIU et al. 2021). In last decades, *P. kudriavzevii* has always attracted increased attentions due to its important role in different food biotechnologies and industrial applications as well as in bio-fertilization, bio-remediation and synthesis of ethanol and glycerol (HERNÁNDEZ-FERNÁNDEZ et al. 2021, SHRUTHI et al. 2022, CHU et al. 2023). Nonetheless, several studies confirmed that *P. kudriavzevii* having a number of virulence factors that enhance it to invading and colonizing the host tissues, and penetrate deep more effectively (KALAIARASAN et al. 2018, JAMIU et al. 2021, DA SILVA et al. 2022).

In Iraq, several fungal pathogens have been isolated from milk of SCM cows (RADHY & SALMAN 2015, JASM MOHAMMED & YASSEIN 2020, JAMEEL & YASSEIN 2021); however, no knowledge on *P. kudriavzevii*. Therefore, this study aims for determining the prevalence of SCM in lactating cows with molecular phylogenetic confirmation of *P. kudriavzevii* in milk of positively infected cows.

MATERIALS AND METHODS

Ethical approval

The current study licensed by the Scientific Committee of the College of Veterinary Medicine (University of Wasit). The collection and examination of milk samples were conducted after oral agreement of the owners of study animals.

Samples

Totally, 400 adult crossbred lactating cows (X±Y DIM) were selected, from rural regions located in Al-Kut City (Wasit, Iraq) during May to June (2021), and subjected to obtaining 50 ml of fresh milk from the available quarters of each animal. The collected samples were transported cooled to the laboratory for CMT and molecular testing.

CMT

Initially, each milk sample of all cows was examined by the CMT-Reagent Kit (Weizur, India) briefly through adding an equal amount of CMT solution to each of milk (3 ml / 3 ml) into a cup that then rotated for two minutes, and the results were interpreted as following:

Color	Score	Result	Liquid reaction
Grey	0	Negative (-)	Clear without any precipitate
Grey / light purple	1	Weak positive (+)	Slight precipitate
Purple	2	Distinct positive (++)	Distinct precipitate with gel-like formation
Dark purple	3	Strong positive (+++)	Distinct gel formation adhere to bottom of a cup

Molecular examination

According to the manufacturer instructions of the Protocol A in the G-Spin^{TM} Total DNA Extraction Kit (Intron Biotechnology, Korea), DNAs were extracted from the positively mastitic milk, and examined by the Nanodrop System (Thermo-scientific, UK) to measurement the purity and concentration of each DNAs sample. Following the manufacturer instructions of the GoTaq® G2 Green Master Mix Kit (Promega, USA), one set of primers (ITSF: 5´- CAA CAA CGG ATC TCT TGG TTC T-3´) and (ITSR: 5´- GCC AAG CGT CCA TGA AAA-3´) was designed based on the GenBank-NCBI (LC413230.1) isolate, manufactured by the Scientific Research Company (Al-Qadisiyah, Iraq), and used to preparing the MasterMix tubes at a final volume of 20µl. The reaction conditions in the Thermal Cycler System (BIO-Rad, USA) were included 1 cycle initial denaturation (95 °C / 5 minutes), 40 cycles of dentauration (95 °C / 30 seconds), annealing (52°C / 30 seconds) and extension (72 °C / 1 minute), and 1 cycle final extension (72 °C / 7 minutes). Electrophoresis of PCR products were done in 1.5% agarose-gel stained with Ethidium Bromide at 100 Volt and 80Am for 1 hour. The product size of positive PCR products was visualized under the UV transilluminator (Clinx, China) at 278 bp.

For phylogeny, eight positive PCR products were sent to the Macrogen Company (Korea) for analysis by the Sanger method. The received data by email were analyzed using the MEGA-X Software, while the multiple sequence alignment was done by phylogenetic tree UPGMA method.

Statistical analysis

The *t*-test in the GraphPad Prism (GraphPad Software Inc., USA) was served to identify significant variation between values of the obtained results at a level of $P<0.05$ (AL-EODAWEE et al. 2023).

RESULTS AND DISCUSSION

An overall 54.25% (217/400) cases were positively reacted by CMT. According to score of positivity, 83.87% (182/217), 11.98% (26/217) and 4.15% (9/217) were showed weak, distinct, and strong positive infections, respectively. In different countries, studies have been conducted to determine the prevalence of organisms causing mastitis. National prevalence rate of SCM was 80% in Mosul (SADOON et al. 2011), 38.89% in Al Sulaimaniyah (HUSSEIN 2012), 68% in Diyala (MINNAT & HAMMADI 2015), and 41.5% in Baghdad and Maysan (SALEEM et al. 2021).

Globally, there were 29% in Algeria (AIT-KAKI et al. 2019), 52.1% in Egypt (ALGAMMAL et al. 2020), 71.02% in Ethiopia (FESSEHA et al. 2021), 73.1% in Kenya (MBINDYO et al. 2020), 30.3% in Nigeria (ANUEYIAGU et al. 2022), 37.7% in China (CHEN et al. 2022), 68.18% in Indonesia (KHASANAH et al. 2021), 65.6-72.3% in Iran (GÓMEZ-QUISPE et al. 2015), 31.4% in Malaysia (SAEED et al. 2022), 42.2% in Pakistan (MAALIK et al. 2019), 54% in Bangladesh (KAHIR et al. 2008), 55.2% in South Korea (SHARMA et al. 2013), 26.9-34.5% in Greece (THEMISTOKLEOUS et al. 2019), 51.28-63% in Turkey (KOÇYİĞİT et al. 2016), 51% in Peru (ALVARADO et al. 2019), 64.9-65.7% in Mexico (PÉREZ-MORALES et al. 2022), 54% in Argentina (DIESER et al. 2014), 23% in Canada (RIEKERINK et al. 2008). Among different studies, obtained results could vary significantly because the method of samples selection, techniques and criteria using when diagnosing a sample, and the role of risk factors. HIITIÖ et al. (2017) mentioned that cow with ≥ 200.000 somatic cells/ml in at least one quarter throughout a year is considered to have SCM; whereas, an existence ≥ 200,000 somatic cells/ml in at least three or all quarters throughout a year to have chronic SCM. In the veterinary practice, researchers demonstrated that fungal infections are responsible for at least 10% of all clinical cases and the almost of these cases are mild (COSTA et al. 1998, KRUKOWSKI 2001, DWORECKA-KASZAK et al. 2012).

Targeting the ITS region, 28.11% (61/217) of samples were reacted positively to *P. kudriavzevii* at 278 bp (Figure 1). Fungal pathogens have been detected largely in fields and pastures; therefore, unhygienic shedding of animals could act as a source of fungal infections to the mammary tissues. In normal issues, the occurrence of fungal mastitis is very low; however, several studies have detected the high prevalence of *P. kudriavzevii* in bovine mastitis when compared to other fungal causes of disease; 45.46% in United Kingdom (GAUDIE et al. 2009), 27.65% in Mexico (ZARAGOZA et al. 2011), 34.6% in Brazil (SARTORI et al. 2014), 32% in Turkey (SONMEZ & ERBAS 2017), and 23.33% in China (DU et al. 2018). The high prevalence *of P.*

kudriavzevii in bovine mastitis might be attributed to their wide existence in environment, high resistant to antifungal therapies, and presence of virulence-related genes (REDDY et al. 2014, GÓMEZ-QUISPE et al. 2015, ZHANG et al. 2019). A number of studies mentioned that *P. kudriavzevii* strains are intrinsically resistant for the first-choice antifungal therapies, and the fast identification of *P. kudriavzevii* will decrease the risk of choice of not correct drugs (ALCAZAR-FUOLI & MELLADO 2014, FORASTIERO et al. 2015, WU et al. 2020). In human, *P. kudriavzevii* has emerged as nosocomial opportunistic fungus which accounts to 2% of candidemic diseases, in particular preference to immunocomprimised patients and those received a large dose of broad-spectrum antibiotics, HIV-protease inhibitors, oral contraceptives, antitumoral agents, and corticosteroids (EGGIMANN et al. 2003a, b). PAL (2023) reported that fungal infections may spread to dairy animals by the milking machine and the milker hands.

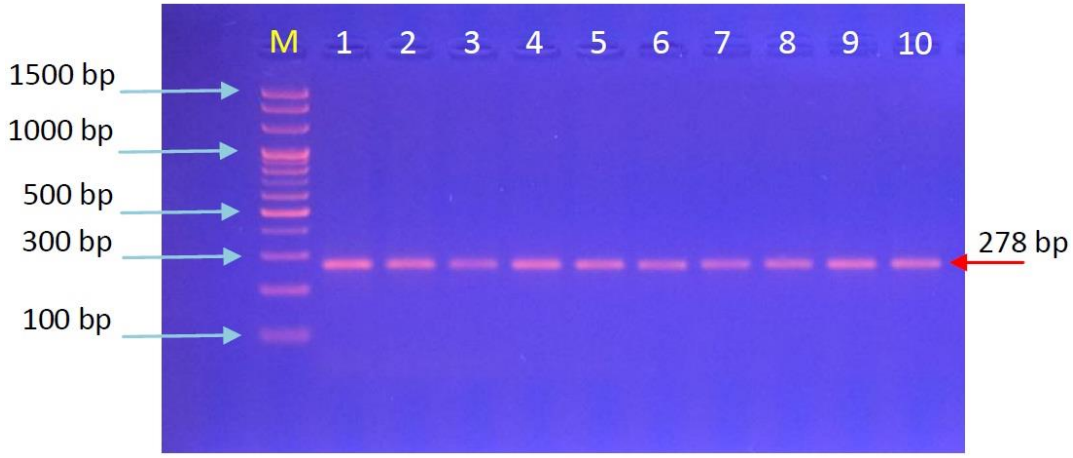

Figure 1. Agarose-gel electrophoresis of some positive *P. kudriavzevii* isolates at 100 Volt and 80Am for 1 hour; Lane (M): Ladder marker (100-1500 bp); Lanes (1-10): Positive local isolates at approximately 278 bp.

Phylogenetic analysis of eight local *P. kudriavzevii* isolates showed the nucleotide alignment similarity (*) and substitutions. Phylogenetic tree analysis revealed that the local *P. kudriavzevii* isolates were showed a genetic identity to the NCBI-BLAST *P. kudriavzevii* Mexican isolates (KY646192.1) at total genetic changes ranged 0.0035-0005% (Table 1, Figure 2).

Table 1. Homology sequence identity between the local and NCBI-BLAST submitted *P. kudriavzevii* Mexico isolate.

Local *P. kudriavzevii* isolate			NCBI-BLAST *P. kudriavzevii* isolate		Identity (%)
No.	Access No.	Size (bp)	Country	Access No.	
1	MZ950631.1	248	Mexico	KY646192.1	99.60
2	MZ950632.1	246	Mexico	KY646192.1	99.59
3	MZ950633.1	244	Mexico	KY646192.1	99.59
4	MZ950634.1	246	Mexico	KY646192.1	99.59
5	MZ950635.1	238	Mexico	KY646192.1	99.58
6	MZ950636.1	246	Mexico	KY646192.1	99.59
7	MZ950637.1	248	Mexico	KY646192.1	99.60
8	MZ950638.1	246	Mexico	KY646192.1	99.59

This study recorded a significant identity between the local *P. kudriavzevii* isolate and the NCBI-BLAST *P. kudriavzevii* Mexican isolates (KY646192.1) that sourced from the vaginal swabs of human samples. In Iraq, lack of molecular information of *P. kudriavzevii* genotypes prevented us to detect evolutionary routes of the species specific lineages or commensal and pathogenic strains, accurately. Hence, we denoting that the local isolates might largely pathogenic and have a role in incidence of SCM in lactating cows.

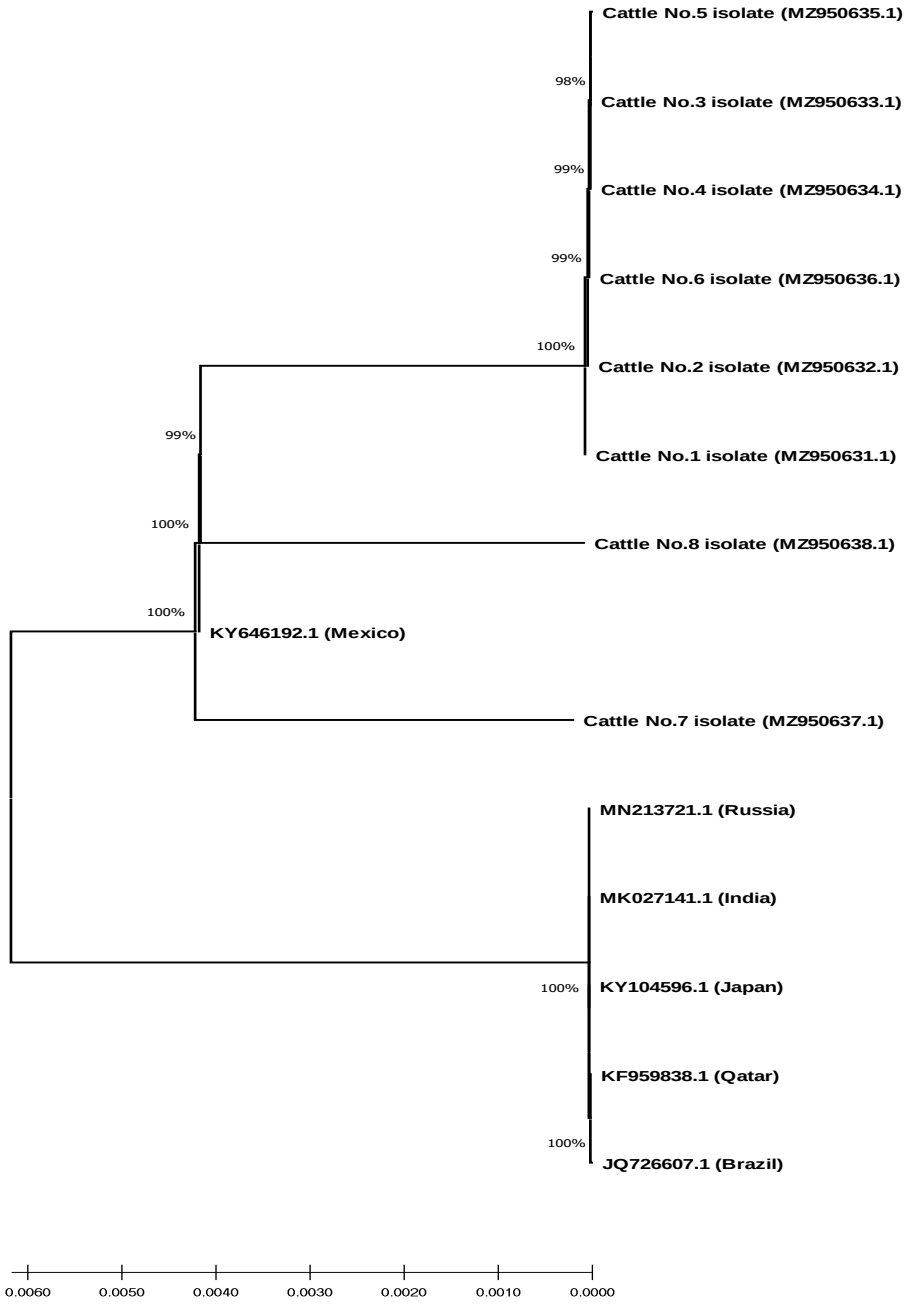

Figure 2. Phylogenetic tree analysis of local and the NCBI-GenBank *P. kudriavzevii* isolates.

DOMÁN et al. (2022) revealed the phylogenetic relationship of *P. kudriavzevii* strains is necessary to understand their ecological lifestyles and the evolution of virulence-associated mechanisms. Moreover, taxonomic, phylogentic and population dynamics reports have demonstrated the importance of this fungus in delineation of ascomycetous yeasts, and substantially to polymorphisms in the ITS region (IWEN et al. 2002, MERSEGUEL et al. 2015). BRILLOWSKA-DABROWSKA & SINIECKA (2012) reported the high specificity of PCR (100%) with the DNA from pure cultures of reference, clinical strains, and human blood samples. Several studies denoting in last decades that the sequencing of rDNA ITS region remains the most reliable tool in rapidly and accurately molecular detection of fungal infections (BEGEROW et al. 2010, RAJA et al. 2017, KULIK et al. 2020). The reasons might be attributed to that the variable conserved rDNA regions have a universal and suitable areas to be used in comparative analysis, clarifying the phylogenetic relationship between species and populations, and identification at taxonomic levels (MERSEGUEL et al. 2015, DOMÁN et al. 2022).

CONCLUSION

This represents the first molecular and phylogenetic study implicated the presence of *P. kudriavzevii* in milk of subclinically mastitic cows. In Iraq, subclinical mastitis remains wide spread among dairy cows

suggesting the need for more active procedures to control or prevent the disease. Worldwide, despite intensive research of clinical mastitis, most subclinical cases of mycotic infections are less established readily. As this fungus is infectious agent, the elaborated DNA extraction procedure and PCR diagnostic assay could be applied in routine laboratory as a confirmative test in case of probable invasive fungal infection. Furthermore, studies should be done to estimate the factor(s) participate in existence and role of this fungus in incidence of mastitis.

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