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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 is a frequent disease with considerable reproductive and economic losses in dairy cattle (PÉREZ-MORALES et al. 2022). This condition is characterized by an inflammatory process in the breast tissue primarily due to intramammary infection or rarely due to physical and chemical etiologies (KUMAR et al. 2020). In the field, intramammary infection is of great importance, as it occurs recurrently by a single or multiple microorganisms, such as algae, bacteria, mycoplasmas, viruses, and yeasts (MOREIRA et al. 2019, RIFATBEGOVIĆ et al. 2024). However, classical mastitis pathogens can be contagious and survive within a host or an environment that opportunistically invades the mammary glands (KIBEBEW 2017, HAIDER et al. 2023)

Based on the presence or absence of clinical signs, this condition is classified as clinical or subclinical, and after the duration of the infection, it is further divided into acute or chronic forms (COBIRKA et al. 2020). In clinical mastitis, abnormal signs in the udder (redness, heat, swelling, solidity and pain) and/or in the 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). Although SCM is characterized by an apparently normal udder without obvious symptoms of udder or milk inflammation, there is a subdetectable decrease in milk production with changes in milk composition, pH, and ion concentration, with obvious elevation in somatic cell levels (UMAM et al. 2017, SALEEM et al. 2021).

In recent years, the occurrence of fungal diseases has increased dramatically in severity in humans and animals due to the increasing number of outbreaks attributed to new species, such as Trichophyton spp. and Aspergillus spp . Candida spp., Geotrichum e Pichia spp., etc. (CARPOURON et al. 2022, PAL 2023). *Pichia kudriavzevii*, formerly known as Candida krusei , is an emerging and unconventional yeast belonging to the family Pichiaceae under the Kingdom of the Fungi Order Saccharomycetales. It was first discovered in 1839 in a patient with typhus (YADAV et al. 2012, JAMIU et al. 2021). In recent decades, *P. kudriavzevii* has attracted increasing attention due to its important role in different food biotechnologies and industrial applications, as well as in biofertilization, bioremediation, and synthesis of ethanol and glycerol (HERNÁNDEZ-FERNÁNDEZ et al. 2021, SHRUTHI et al. 2022, CHU et al. 2023). However, several studies have confirmed that *P. kudriavzevii* has several virulence factors that enhance its ability to invade and colonize host tissues and penetrate more effectively (KALAIARASAN et al. 2018, JAMIU et al. 2021, DA SILVA et al. 2022).

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

MATERIALS AND METHODS

Ethical approval

The current study was approved by the Scientific Committee of the Faculty of Veterinary Medicine (Wasit University). The collection and examination of milk samples were performed after an oral agreement from the owners of the study animals.

Samples

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

CMT

Initially, each milk sample from all the cows was briefly examined using a CMT Reagent Kit (Weizur, India) by adding an equal quantity of CMT solution to each milk (3 ml/ 3 ml) in a beaker, which was then rotated for 2 min, and the results were interpreted as follows:

Color	Score	Result	Liquid reaction
Gray	0	Negative (-)	Clear without precipitation
Gray/light purple	1	Weak positive (+)	Slight precipitation
Purple	2	Distinct positive (++)	Distinct precipitate with gel-like formation

Dark purple	3	Strong positive (+++)	Distinct gel formation on the glass bottom

Molecular examination

According to the manufacturer's instructions for Protocol A in the G-Spin ™ Total DNA Extraction Kit (Intron Biotechnology, Korea), DNA was extracted from positive mastitic milk and examined using a Nanodrop System (Thermo-scientific, UK) to measure the purity and concentration of each DNA sample. Following the manufacturer's instructions for the GoTaq® G2 Green Master Mix kit (Promega, USA), a primer set (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 isolate (LC413230.1), manufactured by Scientific Research Company (Al-Qadisiyah, Iraq) and used to prepare MasterMix tubes in a final volume of 20µl. The reaction conditions in the Thermal Cycler System (Bio-rad, USA) included 1 cycle of initial denaturation (95 °C/5 min), 40 cycles of declaration (95 °C/30 s), annealing (52 °C/30 s), and extension (72 °C/1 min), and 1 cycle of final extension (72 °C/7 min). The electrophoresis of the PCR products was performed on a 1.5% agarose gel stained with ethidium bromide at 100 V and 80 mA for 1 h. The product size of the positive PCR products was visualized using a UV transilluminator (Clinx, China) at 278 bp.

For phylogeny, eight positive PCR products were sent to Macrogen Company (Korea) for analysis using the Sanger method. Data received by email were analyzed using MEGA-X software, and multiple sequence alignment was performed using the UPGMA method of the phylogenetic tree.

Statistical analysis

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

RESULTS AND DISCUSSION

In general, 54.25% (217/400) of the patients reacted positively to CMT. According to the positivity score, 83.87% (182/217), 11.98% (26/217), and 4.15% (9/217) had weak, distinct, and strong positive infections, respectively. In different countries, studies have been conducted to determine the prevalence of mastitis-causing organisms. The national prevalence rates of MCS were 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).

Overall, 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 Türkiye (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, the results obtained may vary significantly due to the sample selection method, the techniques and criteria used when diagnosing a sample, and the role of risk factors. HIITIÖ et al. (2017) mentioned that a cow with $\geq$ 200,000 somatic cells/ml in at least one quarter over a year is considered to be a carrier of SCM; whereas the existence of $\geq$ 200,000 somatic cells/ml in at least three or all quarters over a year has chronic SCM. In veterinary practice, researchers have demonstrated that fungal infections are responsible for at least 10% of all clinical cases, and almost all 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 the samples reacted positively to *P. kudriavzevii* at 278 bp (Figure 1). Fungal pathogens are detected mainly in fields and pastures; therefore, unhygienic animal waste may act as a source of fungal infections in mammary tissues. In normal cases, the occurrence of fungal mastitis is very low; however, several studies have detected a high prevalence of *P. kudriavzevii* in bovine mastitis when compared to other fungal causes of the disease; 45.46% in the United Kingdom (GAUDIE et al. 2009), 27.65% in Mexico (ZARAGOZA et al. 2011), 34.6% in Brazil (SARTORI et al. 2014), 32% in Türkiye (SONMEZ & ERBAS 2017), and 23.33% in China (DU et al. 2018). The high prevalence *of P. kudriavzevii* in bovine mastitis can be attributed to its widespread existence in the environment, high resistance to antifungal therapies, and the presence of virulence-related genes (REDDY et al. 2014, GÓMEZ-QUISPE et al. 2015, ZHANG et al. 2019). Several studies have mentioned that *P. kudriavzevii* strains are intrinsically resistant to first-line antifungal therapies, and rapid identification of P. kudriavzevii decreases the risk of incorrect drug selection (ALCAZAR-FUOLI & MELLADO 2014, FORASTIERO et al. 2015, WU et al. 2020). In humans, *P. kudriavzevii* has emerged as a nosocomial opportunistic fungus responsible for 2% of candidemia diseases,

with a particular preference for immunocompromised patients and those receiving a large dose of broad-spectrum antibiotics, HIV protease inhibitors, oral contraceptives, antitumor agents, and corticosteroids (EGGIMANN et al. 2003a, b). PAL (2023) reported that fungal infections can spread to dairy animals via the milking machine and the milker's hands.

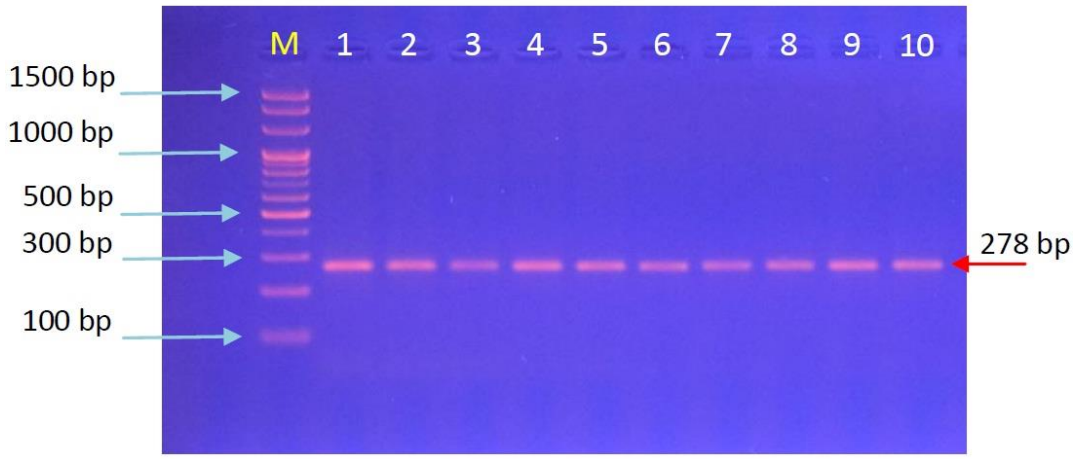

Figure 1. Agarose gel electrophoresis of some positive isolates of *P. kudriavzevii* at 100 V and 80 mA for 1 h; lane (M): Ladder marker (100-1500 bp); bands (1-10): Local positive isolates at approximately 278 bp.

Phylogenetic analysis of eight local isolates of *P. kudriavzevii* revealed similar nucleotide alignments (*) and substitutions. Phylogenetic tree analysis revealed that local *P. kudriavzevii* isolates exhibited genetic identity with Mexican NCBI-BLAST isolates of *P. kudriavzevii* (KY646192.1), with total genetic changes ranging from 0.0035% to 0.0005% (Table 1, Figure 2).

Table 1. *Homology sequence identity of the local P. kudriavzevii isolate from Mexico submitted to NCBI-BLAST.*

Local isolate of *P. kudriavzevii*			*Isolate NCBI-BLAST of P. kudriavzevii*		Identity (%)
No.	Access number	Size (bp)	Country	Access number	
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 identified a significant identity between the local *P. kudriavzevii* isolate and the Mexican NCBI-BLAST *P. kudriavzevii* isolate (KY646192.1) from vaginal swabs of human specimens. In Iraq, a lack of molecular information on *P. kudriavzevii* genotypes prevented us from accurately detecting the evolutionary pathways of species-specific lineages or commensal and pathogenic strains. Therefore, the local isolates may be broadly pathogenic and may play a role in the incidence of SCM in lactating cows.

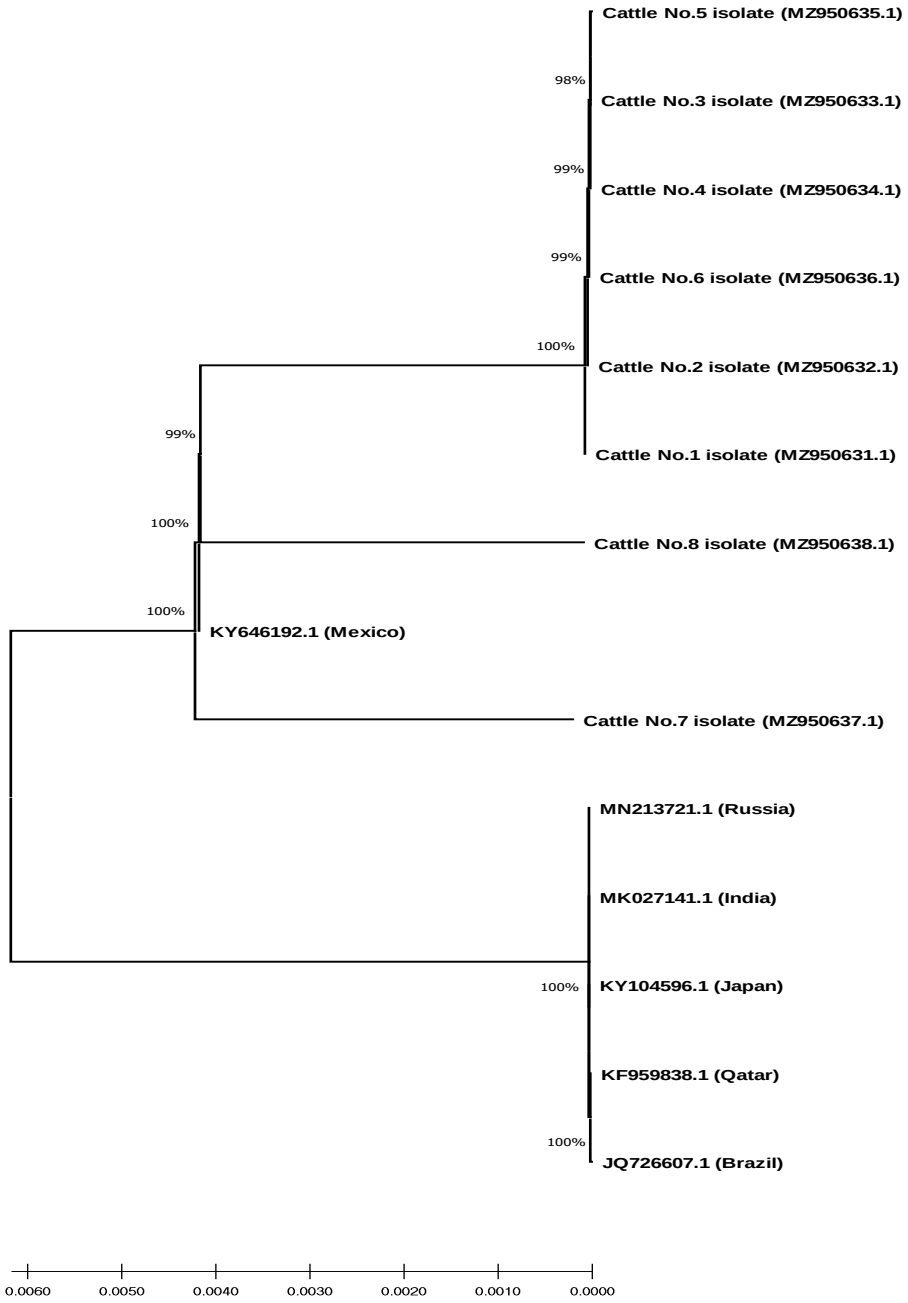

Figure 2. *Analysis of the phylogenetic tree of local P. kudriavzevii strains from NCBI GenBank*

DOMÁN et al. (2022) revealed that the phylogenetic relationships among *P. kudriavzevii* strains are necessary for understanding their ecological lifestyles and the evolution of mechanisms associated with virulence. Furthermore, taxonomic, phylogenetic, and population dynamics reports demonstrated the importance of this fungus in the delineation of ascomycete yeasts and, substantially, in 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 DNA from pure reference cultures, clinical strains, and human blood samples. Several recent studies have indicated that sequencing of the ITS region of rDNA remains the most reliable tool for rapid and accurate molecular detection of fungal infections (BEGEROW et al. 2010, RAJA et al. 2017, KULIK et al. 2020). The reasons can be attributed to the fact that conserved rDNA variable regions have universal and suitable areas to be used in comparative analyses, clarifying the phylogenetic relationships between species and populations, and identifying taxonomic levels (MERSEGUEL et al. 2015, DOMÁN et al. 2022).

CONCLUSION

This is the first molecular and phylogenetic study to implicate the presence of *P. kudriavzevii in the milk of subclinically mastitic cows* . In Iraq, subclinical mastitis remains widespread among dairy cows, suggesting

the need for more active control or prevention procedures. Despite intensive research into clinical mastitis, most subclinical cases of fungal infections are less easily established. Because this fungus is an infectious agent, an elaborate DNA extraction procedure and PCR diagnostic assay can be applied in the routine laboratory as a confirmatory test in cases of probable invasive fungal infection. Furthermore, studies should be conducted to estimate the factor(s) that participate in the existence and role of this fungus in the incidence of mastitis.

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