Short Communication

Isolation and Identification of Fungi from Reproductive Tract of Healthy Bali Cows

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Abstract | Bacterial and fungal diseases, such as mycotic endometritis and abortion, are one of the causes of reproductive diseases in Bali cattle. Earlier studies on bovine uterine microflora have mainly focused on bacteria; however, the presence of fungi has yet to be discussed in detail. This study aimed to provide data on the prevalence and identification of fungi by using morphological methods. The samples were vaginal swabs obtained from 22 healthy female Bali cattle from various locations in Bali, Indonesia. For isolation, vaginal swabs were cultured on Sabouraud's dextrose agar (SDA) medium and identification was performed macroscopically by observing colony growth and microscopically using the tape smear method stained with Methylene Blue stain. The results showed that 32 fungi belonging to seven genera were isolated from 22 Bali cows. The prevalence rates of *Aspergillus, Fusarium, Cladosporium, Curvularia,* and *Candida, Mucor,* and *Penicillium* spp. were 41, 25, 19, 6, and 3%, respectively. These data add important information to the literature on the fungal microbiota in healthy Bali cows.

Keywords | Fungi, Bali Cattle, Vaginal swab, Morphology Identification, Mycotic

Received | May 23, 2023; **Accepted** | June 20, 2023; **Published** | November 01, 2023
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Citation | Sudipa PH, Mahatmi H, Gelgel KTP, Suarjana IGK, Besung INK, Mufa RMD, Sukernayasa IW (2023). Isolation and identification of fungi from reproductive tract of healthy bali cows . J. Anim. Health Prod. 11(4): 337-343.
DOI | http://dx.doi.org/10.17582/journal.jahp/2023/11.4.337.343
ISSN | 2308-2801

INTRODUCTION

Bali cattle are iconic animals from Bali Island and are the best choice for livestock farmers among several breeds. Bali cattle have unique characteristics such as red brick color on females and adult males with a blackish color of fur, white on the lower limbs, the back of the pelvis (buttocks), and good reproduction performance (Merdana et al., 2020). The reproductive health of Bali cattle is also critical for their preservation (Puja et al., 2018). Abortion, especially in cattle, is fundamental to study because it is one of the factors inhibiting the government's plan to increase the cattle population to meet the demand for meat consumption (Gholib and Ahmad, 2013). Reproductive disorders can be caused by various microorganisms, including bacteria (*Brucella spp.*, *Vibrio spp.*, and *Staphylococcus spp.*), viruses, mycoplasma, and fungi (Hariadi et al., 2011). Bacterial and fungal microflora that inhibit the reproductive tract are among the causes of abortion. In Indonesia, of the 20.4% of cases of reproductive disorders in dairy cows, 2-5% are thought to be caused by bacterial infections, while those caused by fungal infections are not

yet quantified (Hariadi et al., 2011).

According to Wienanto (2008), cases of mycotic abortion are mainly caused by *Aspergillus fumigatus* and *Mucor* species. The infection resulting in abortion begins with the entry of fungal spores into the animal's body through the respiratory and digestive organs, which are then carried to the placenta through the bloodstream, causing inflammation in pregnant cattle and deteriorating fetal growth. From an ecological point of view, host microflora are not separate environments but are a network of interconnected communities that are continually exchanging (Neckovic et al., 2020). Therefore, microorganisms can enter the reproductive tract from other anatomical sites; for example, at birth, the physical cervical barrier is decreased, allowing microorganisms to be transported from the vagina, feces, animal skin, or from the environment via the vagina to the genital tract (Piersanti and Bromfield, 2019). Studies of the postpartum bovine uterine microflora have mainly focused on bacteria, but the presence of fungi has also been mentioned, although not discussed in detail (Bonnet et al., 1991). According to Ahmed and Bhattacharyya (2015), the prevalence of pathogenic fungi in dairy cows and buffaloes was reported at 17.98%, and the most common isolated fungi were *Aspergillus fumigatus* and *Penicillium spp.* in cattle and buffalo. Derakhshandeh et al. (2015) reported 8.7% (25–35 days postpartum) and 5.5% (39–49 days postpartum) prevalence of mycotic endometritis in Holstein cattle, and the most frequently isolated fungus was *Aspergillus spp.* (60.0%), *Penicillium spp*. (26.0%), and Yeast (13.0%).

There is a growing interest in the microflora of the reproductive tract, and its relationship with disease and health is a new and rapidly evolving field of study. Although the microbiome can have a significant influence on host biology, relatively little is known about microbial communities in the reproductive tracts of dairy and beef cattle (Appiah et al., 2020). Because of the large number of fungal infections that can cause reproductive disorders in cattle and the absence of initial data on the prevalence and identification of fungi in the reproductive tract, especially in female Bali cattle, it is necessary to identify the fungus and the prevalence of fungi inhabiting the reproductive tract of Bali cattle.

MATERIALS AND METHODS

Sample collection

Vaginal swabs were aseptically collected from female Bali cattle (n = 22) under normal health conditions. Sterile swabs (without transport media) were used for sample collection. Samples were collected in triplicates. The cows selected for sample collection were from various locations in Bali province, Indonesia. General data such as age, weight and sample location were recorded during sample collection.

Isolation and identification procedures

Sabouraud's dextrose agar (Oxoid CM41) medium was prepared according to manufacturer instructions (Oxoid, UK). In brief, sixty-five grams of Sabouraud's dextrose agar was mixed with 1000 ml of distilled water and put into an Erlenmeyer. The solution was homogenized using a digital magnetic stirrer. The Gentamicin antibiotic (40 mg/ml) was added to the medium to prevent the bacterial growth. The media was sterilized using an autoclave for 15 minutes at 121C° and then poured into the Petri dishes. Then the vaginal swabs were seeded to the media and plates were incubated at 25 C° for 3 days. After 3d, the yeast were sub-cultured to get pure culture for identification.

Fungi identification was conducted both macroscopically and microscopically. The macroscopic examination was carried out by observing the presence of fungal growth in a petri dish and confirmed directly with the naked eye. The microscopic examination was made by slide preparation to see under the microscope for the hypha, sporangia, oogonia and reproduction structures (Yudiarti et al., 2012). The tape smear method using Methylene Blue staining was used for microscopic identification.

Data Analysis

The data were tabulated and explained descriptively. The prevalence percentages of various fungal organisms was expressed in percentages.

RESULTS AND DISCUSSION

Thirty-two fungi belonging to seven genera were isolated from vaginal swabs of Bali cattle (Table 1). The isolated fungal organisms included *Aspergillus, Fusarium, Cladosporium*, and *Curvularia* spp., with prevalence rates of 41, 25, 19, and 6%, respectively. *Candida, Mucor*, and *Penicillium* spp. were also found, with prevalence rates of 3% each.

Table 1: The Prevalence of Fungi in the reproductive tract of healthy Bali cows.

Isolated Fungi	Number of isolates	Prevalence (%)
Aspergillus spp.	13	41
Fusarium spp.	8	25
Cladosporium spp.	6	19
Curvularia spp.	2	6
Candida spp.	1	3
Mucor spp.	1	3
Penicillium spp.	1	3
	32	100

Aspergillus species

Aspergillus spp. are considered environmental fungi that are transmitted by airborne conidia. Their colony could be observed as white, yellow, green, red, or black molds (Kaur, 2017). Identifying characteristics of *Aspergillus sp*p. include the size and arrangement of conidial heads, size, and shape of vesicles, and phialide arrangement, as well as the color, size, and length of spores produced (Kaur, 2017). The *isolated Aspergillus spp. formed* a black-colored colony Fig. 1a) and macroconidia that were microscopically observed in long dry chains that may diverge or coalesce in compact columns (Fig. 1b) (Saleemi et al., 2017). *Aspergillus* species were found in abnormal cervical mucus discharge of Holstein-Friesian cows and heifers with a 4% incidence rate (Ata et al., 2010). In another study, *Aspergillus fumigatus* was the most commonly isolated fungi in repeat breeding cows and buffaloes, with 17.98% prevalence rate (Ahmed & Bhattacharyya, 2015). Nevertheless, in this current study used healthy cows, most likely, the fungi that were isolated are contaminants from the soil, animal excreta, and even in the vegetative parts of the plants or feed waste. The fungi may also enter the reproductive tract of animals most commonly from skin or feces (Stout, 2008).

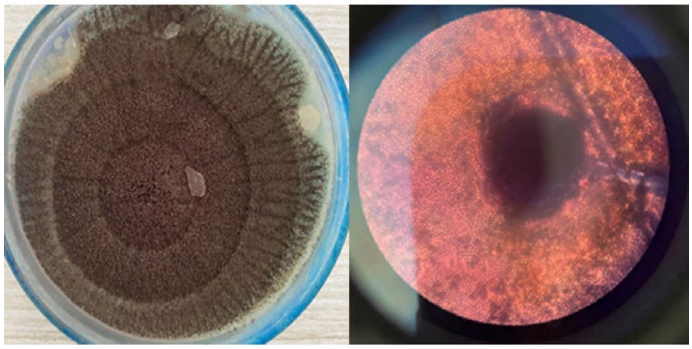

Figure 1: *Aspergillus* colony (a), and microscopic macroconidia (b).

Fusarium species

The genus *Fusarium* consists of more than 80 species of fungi, which can infect plants, vertebrates, insects, humans, or even other fungi (Ivic et al., 2009). It is the most diverse group that can infect even food and feed grains. *Fusarium* species produce long, multicellular, canoe-shaped, or banana-shaped macroconidia. These large asexual conidia are the morphological characteristic of the genus. Many species also produce small, generally single-celled microconidia ranging from fusiform to oval to spherical shapes (Glenn, 2007). Colonies proliferate and may be pale (whitish to cream) or brightly coloured in yellow, brownish, pink, reddish, violet, or lilac shades (Abdel-Azeem et al., 2019). The isolated fungi exhibited pale white colonies (Fig.2a) with banana-shaped macroconidia (Fig.2b) as the definitive characteristic (Glenn, 2007; Abdel-Azeem et al., 2019). Furthermore, *Fusarium* is a typical soil-borne genus, widely distributed and generally abundant in all types of soils around the world (Backhouse et al., 2001). These vaginal swab samples showed *Fusarium spp.*, most likely because the vagina has contact with the contaminated soil in the surrounding cowshed. High concentration of these airborne spores is usually associated with working environments and tasks, like uploading of grain or hay (Jestoi, 2008). This fungi is called as "storage fungi" because it grown in stored grains when adequately dried prior to storing, or stored in high humid environments. The taxa that are most importantly included in the storage pathogen category are those belonging to the genera of *Aspergillus* and *Penicillium* (Magan et al, 2010).

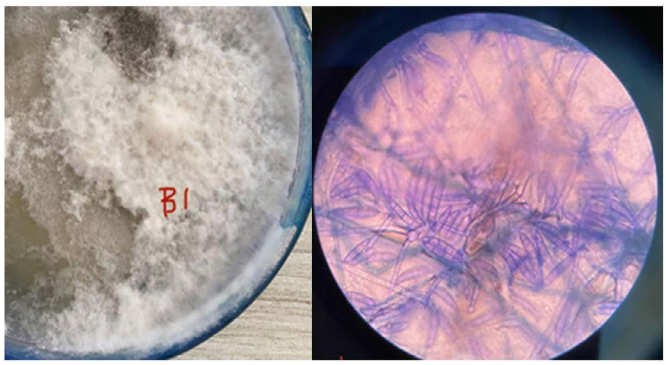

Figure 2: *Fusarium* colony (a), and microscopic macroconidia (b)

Cladosporium species

The genus *Cladosporium* is hygiene standard in many parts of the world; their spores can be found in air, soil, and water (Domsch et al., 1980). Some species are described as a cause of opportunistic phaeohyphomycosis, including subcutaneous and deep infections in humans and animals (Sandoval-Denis et al., 2015). *Cladosporium* morphology showed solitary to fasciculate conidiophores, proliferating, sympodial, and forming unbranched or branched acropetal conidial chains (Bensch et al., 2012). The colony characteristic is grey-green to deep green, flat or folded, velvety to dusty or granular, aerial mycelium scarce, sometimes showing cottony to floccose white to grey cushions, with a regular margin; reverse dark green to black (Sandoval-Denis et al., 2016). The current research showed that the fungi have a dark green colony (Fig.3a), and the microscopic findings represent branched and chain-type conidia (Fig.3b). These characteristics match *Cladosporium* fungi morphology in the previous research (Bensch et al., 2012).

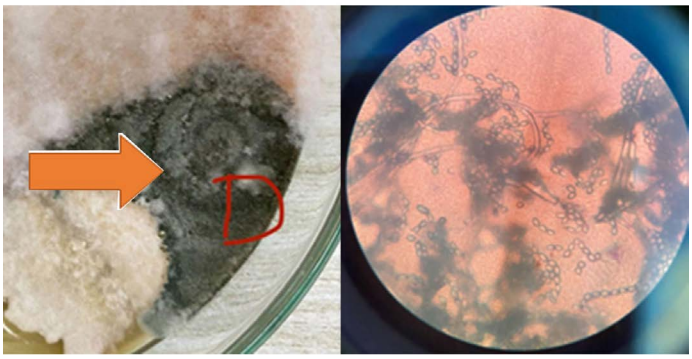

Figure 3: *Cladosporium* colony (a), and microscopic appearance of *Cladosporium* spp. (b)

Curvularia species

This fungus is a ubiquitous pigmented fungus that is usually recovered from vegetative matter and soil (Domsch et al., 1980). The color of the fully grown *Curvularia* colonies was dark brown to black on the obvious side. On the reverse side, the colonies appeared black. The texture of the colonies was hairy and, to some extent, woolly, and the colonies appeared to be slightly raised (Subapriya et al., 2015). *Curvularia* microscopically shows the characteristics of brown hyphae and brown conidiophores and produces branched septa. As the *Curvularia* fungus matures, one of the central cells in the conidia grows larger and darker than the lateral cells, which produces a characteristic curvature and gives it a croissant-like appearance (Paterson & Lima, 2015). Current research result is related to previous findings that showed that macroscopy colony of *Curvularia* have a characteristics of hairy and dark brown to black color (Fig.4a). The microscopic examination also matches the characteristic of general *Curvularia sp*p. with branched septa and curvature croissant-like conidia (Fig. 4b).

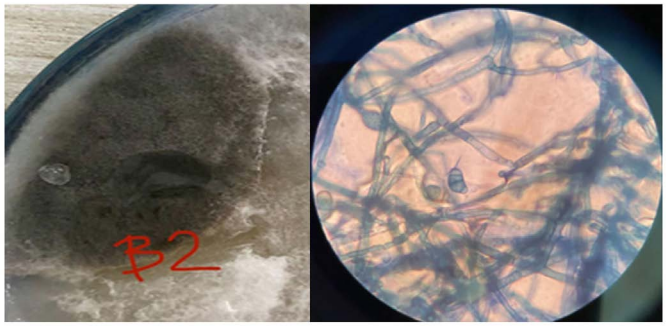

Figure 4: *Curvularia* colony (a), and microscopic appearance of *Curvularia* spp. (b)

Candida species

As a saprophyte, Candida can be found on the skin, genital tract, upper respiratory tract, and digestive tract, including the oral cavity (Zaremba et al., 2006). One of the species of this fungi, *Candida albicans*, are important opportunistic yeast identified as the cause of endometritis in water buffalo (Pal, 2002). *Candida sp*ecies colonies are white to cream in color, smooth, and shaped like yeast (Hamid et al., 2014), matching the result in this research, which shows the growth of white-cream colonies with yeast-like shapes. According to Yilmaz et al. (2012), the uterine culture showed 12.96% *Candida spp*. in healthy Anatolian Water Buffaloes. However, in this research, only 3% of *Candida sp*p. are isolated. *Candida* are opportunistic fungi, occurring as normal inhabitants of the digestive tract, oral cavity, and vagina of humans and many of our domestic animal (Chengappa et al, 1984). The difference in prevalence rate in our and previous researches might be due to the difference in degree of colonization of these fungi in different animal species/breeds.

Mucor species

These fungi can be easily isolated from soil, dung, water, stored grains, and plants (Nguyen et al., 2016). These fungi also play the role of endophytic fungi; it has a round colony shape and flat colony margins, the surface of the colony is fine fibrous like thick cotton, the mycelium is white, the color of the colony on the front surface, and the color on the back surface is white. At the beginning of growth, the mycelium is white, and it turns black after the spores appear (Yuliana, 2016). *Mucor sp*p. has a branched sporangiophore (sympodial or monopodial), and the columella is pear-shaped, round, or elliptical. Sporangiospores are elliptical to semi-spherical in shape and have a 5 – 10 µm diameter. This species can grow to sporulate at temperatures of 5 – 20°C but not at 37°C (Gandjar, 1999). In this research, the fungi that were isolated has a similar characteristic to that of *Mucor sp*p. with white to the black colony and ellipse or round microscopic sporangiospores appearance (Figure not showed).

Penicilium species

The occurrence of this fungi in cervicovaginal fluids of Holstein dairy cows has been observed from the cervix and vagina at 27.14% and 28.57% respectively in infertile/repeat breeder cows (Garoussi et al., 2007). However, in this research, the samples were taken from healthy Bali cattle with no reproductive problems, but this finding should become attention to farmers. Specific characteristics of *Penicillium* are insulated or septate hyphae, branched mycelium, usually colorless, conidiophores insulated or septate and appear above the surface originating from hyphae below the surface of branched or unbranched hyphae, the head of the hyphae carrying spores is shaped like a broom, with sterigmata appear in clusters, conidia are chain-shaped because they arise one by one from the sterigmata and for their conidium, when young color is green, then turns bluish or brown (Fardiaz, 1992). *Penicillium spp.* on the SDA medium had septate hyphae. Conidia are spherical. Initially white, then changed to blue-green, greenish grey, and the opposite color is usually pale yellow. At the same time,

the microscopic form of the fungus *Penicillium* has hyphae, round conidia, and a collection of phialides (Ristiari et al., 2018). In this research, the characteristic of the *isolated Penicillium fungi* was similar and has a green-colored colony with conidia forming in chains when observed in a microscope (Figure not shown).

Fungi contamination can be pathogenic that excrete some pathogen protein to infect the host and toxigenic fungi that produce toxin for their virulence, but not all molds are classified as that type. Pathogenic molds include, among others, *Aspergillus* (*A. fumigatus, A. terreus, A, nidulans, A. niger., Mucor spp., Absidia spp., Trichoderma spp.* and others). In contrast, toxigenic molds include *A. flavus, Penicillium spp., Fusarium spp.*, and others (Ahmad & Gholib, 2017). However, in this research, the Bali cattle were healthy and have no reproductive system issues. Many of the isolated fungi are environmental fungi that can be easily found in soil, air, or feed close to the cow shed. Various predisposing factors help the fungus to cause infection in the reproductive tract, the exact conditions that make the fungus capable of colonizing the uterus or reproductive tract are still unknown (Stout, 2008). Although the primary reservoir of the fungal pathogens is the caudal reproductive tract, sometimes contamination from feces may also occur due to neovagina (Dascanio, 2000). It is presumed to cause ascending reproductive tract infection (Zafracas, 1975), and due to the opportunistic nature of the fungal pathogen, disease results when it accidentally penetrates host barriers and during immunologic deficiency or debilitating conditions which helps the fungus to enter and grow inside the host (Hogan et al., 1996).

Feed and fodder are also significant sources of contamination. Mycotoxins are secreted from *Aspergillus* and *Penicillium* that grow on foods in humid weather (85%) and temperatures between 12°C and 25°C. Fungal spores are found in good-quality fodder material at 10^6/g but more in low-quality fodder (Laing et al., 1988). Such contaminated food material can act as a direct or indirect source of fungal pathogens to the bovines. With the findings of high *Aspergillus sp*p. prevalence, the farmers and related parties should be concerned about this research result. However, the function of these opportunistic fungal pathogens, which play a role in the various processes, requires further investigation. The microflora in the cattle vaginal tract has also recently been revealed to play a significant role as biomarkers of reproductive success and failure (Deng et al., 2019).

CONCLUSION

Morphological identification of fungi isolated from the Bali cattle reproductive tract showed that the genus *Aspergillus* was dominant, followed by *Fusarium, Cladosporium*, and *Curvularia*, while *Candida, Mucor*, and *Penicillium* species had the lowest prevalence. These findings add important information to the literature on the fungal microbiota in healthy Bali cows. In the future, more advanced techniques such as molecular methods for precise identification should be adopted.

ACKNOWLEDGEMENT

The authors thank the Udayana University Research and Community Services, Laboratory of Bacteriology and Mycology, Faculty of Veterinary Medicine, and fellow veterinarian colleagues for providing research facilities and support.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

NOVELTY STATEMENT

The morphological identification and types of Bali cattle reproductive tract fungi have yet to be researched. With many possibilities of fungal disease that can occur to the Bali cattle, this data offers basic information as stepping stone for the following research.

AUTHORS CONTRIBUTION

Putu Henrywaesa Sudipa and Romy Muhammad Dary Mufa conducted the research process in the laboratory. Hapsari Mahatmi, Ketut Tono Pasek Gelgel, I Gusti Ketut Suarjana, and I Nengah Kerta Besung designed the research and provided media for the research. I Wayan Sukernayasa, helped in the data collection process.

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