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Identification of Potentially Pathogenic Yeast Species in Seagull Guano by Molecular Techniques

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Abstract:

Although studies have been performed identifying bacteria present in seagull guano, limited studies have been done with fungi, leaving a significant gap in our knowledge of a potentially significant reservoir of human disease. If pathogenic fungi are being deposited by seagulls in their feces then it is possible that the Department of Health might elect to monitor sand, as well as water, for a broad spectrum of disease-causing microbes. Currently only water is tested for the presence of coliform bacteria. It is hypothesized that there are pathogenic yeast-like fungi present in the guano of seagulls, that these fungi are deposited in areas around Myrtle Beach. These fungi may present a potential health risk to beach-goers. Six samples were collected from solid surfaces at randomly chosen public beach accesses within the Myrtle Beach city limits. These samples were then washed, diluted, and plated on Bengal Rose agar, which enhances fungal growth, and incubated at 37 degrees Celsius. Discrete colonies were then selected and grown in Sabouraud Dextrose broth at 37 degrees Celsius. Following this step DNA was isolated from the fungal cultures and polymerase chain reaction was performed, using universal fungal primers to amplify the ITS-5.82S-ITS2 region of the rDNA, which yielded three PCR products for sequencing. The sequencing reactions were unsuccessful but it was apparent that seagull guano acts as a carrier for fungi whose presence could impact the health of beach-goers. The relative significance of this source, as well as the other potential sources, may lend suggestions for controlling the presence of potentially pathogenic fungi on recreational beaches to public health officials.

Introduction:

Fungi are classified in the Kingdom Fungi, which have general characteristics such as absorptive nutrition, cell walls that contain chitin, and reproduction both sexually and asexually through spores. Within this classification, individual species are differentiated by the type of sexual and asexual spores, the presence or absence of fruiting structures, the morphology of the somatic cells, nutritional preference and ecological niche (Madigan et al. 2009). Many pathogenic fungi are dimorphic and can take on a second form at higher temperatures (e.g. 37°C), while some species of fungi exist only as single-celled yeast. The organisms that this study specifically focused on are found as yeast or produce yeast-like cells at 37°C which is the average human body temperature (Webster and Weber 2007).

As of 2006, at least 100 species of fungi have been identified as being pathogenic towards humans (Moace et al. 1990). In most cases pathogenic fungi infect individuals who are immunosuppressed. Individuals who are considered immunosuppressed could be undergoing chemotherapy, taking immunosuppressant drugs for transplants, taking long courses of antibiotics or have diseases such as AIDS. As early as 1987 the CDC proposed that mycoses, or fungal diseases, should be included as a diagnostic criterion for AIDS (Leaw et al. 2006). Elderly individuals and young children are also considered to be immunosuppressed as elderly individuals have an immune system weakened by time, and children have immune systems that are not yet fully developed (Rees and Dixon 1981). Most fungal organisms are introduced to the human body either through abrasions or lacerations to the skin (Bergen and Merner 1977) Some organisms also enter the body through respiratory routes, when spores are inhaled.

Fungi that cause mycoses are found in a variety of environments such as sand, soil and fecal matter (Bergen and Merner 1977) (Buck 1983) (Cragg and Clayton 1971). Yeast species deposited by humans have been shown to have a direct correlation between their abundance in an

environment and the number of humans present at any given time (Buck 1983). This correlation suggests that humans can act as vectors of fungi, or that other animals may be attracted to high populations of humans and that they act as the vectors. Studies have shown that there is an abundance of fungi present in sand in beach areas. Vogel and his colleagues found, as expected, that there was a high occurrence of yeast species, because sand can filter microbes from the water during tidal cycles. They hypothesized that these fungi could withstand the high salinity and high heat in this environment because humans carry them. In their study, over twenty-one species of fungi were isolated from the sand samples, including three species that were found in every sampling (Buck 1983). It can be concluded from this study that pathogenic fungi can be present in the sand on beaches. It is also plausible that the sand may not be the only source for pathogenic fungi on the beach.

A second source, important to this current study, is the presence of fungal pathogens in the fecal matter of birds, particularly seagulls. Studies have shown that several birds carry fungal pathogens in their fecal matter, as evidenced by the presence of *Cryptococcus neoformans* in samples of Canadian geese (Filion et al. 2006). *Candida tropicalis* is known to be a common human pathogen and is found in the intestines of most gulls, in Vogel's study, *Candida tropicalis* was isolated from the sand on the beach (Buck 1983) suggesting that gulls could contribute the fungus to the sand. In another study *C. neoformans* was isolated from fecal matter found on the sand of Oahu, Hawaii (Kishimoto and Baker 1969). Cragg and Clayton's study showed that over 25 different species of fungi were isolated from the fresh and dry samples of seagull guano (Kishimoto and Baker 1969). They hypothesized that the fungi did not originate from the food the gulls were consuming due to the lack of common bacteria found in decaying matter, but rather originated as natural flora within the gulls' digestive tract. It has been established that bird

guano (including seagull) contains potential fungal pathogens and it has also been demonstrated that fungal pathogens can be isolated from beach sand. What has not be established is that the fungal pathogens in the sand are of seagull origin and more importantly, what the health risk is of these fungal pathogens to people who use these beaches.

Traditionally identification of fungi has been based upon morphological characteristics of colonies that form on plates followed by microscopic examination. For example a colony that is pink and shiny in appearance is generally a different species from one that is green and filamentous. Then based upon microscopic examination of the types of hyphae present as well the sexual spores produced, the species can be determined. However, with the advent of new molecular techniques fungi can be identified using their genomic DNA. Polymerase chain reaction (PCR) techniques are used to amplify specific regions of DNA. This is accomplished by using universal primers. Universal primers anneal to a well-conserved region of DNA, generally the interspatial regions (ITS) of ribosomal DNA. The region between the two annealing sites is amplified and varies between species. The product obtained from this PCR step can then be sequenced and compared to known sequences using the Basic Local Alignment Search Tool or BLAST. BLAST is a tool produced and run through the National Center for Biotechnology Information (NCBI), which is part of the National Library of Medicine and the National Institute of Health. BLAST is used to enter unknown sequences of nucleotides or amino acids and is then compared to stock sequences of known organisms to find the closest match. BLAST will rank all results based upon similarity and in most cases identify the species and if not specifically the species, the genus.

The rationale for studying the presence of these potentially pathogenic fungi is to identify and quantitate those species present, in order to provide information of potential use to those

state agencies charged with monitoring and maintaining a healthy environment at recreational beaches. Currently there is no international agreement on the amount of fungal propagules (or biomass) that is considered unsafe for a particular environment (Koehn 1982). In the United States, most state agencies do not test for the presence of fungi in areas unless there is some epidemiological data that warrants concern. A study performed in the last ten years correlated the presence of fungi in locations with the number of swimmers present at the time of sampling (Koehn 1982). This study again indicates that there is a higher correlation between the presence of humans and the population of fungi found in an environment. As a result young children, the elderly, and other immunosuppressed people may be at risk for infection. Gulls often feed off of the trash associated with human use and are highly abundant on heavily-used beaches. Gulls produce about 50 grams of wet feces per day, which can contain up to 3×10^{10} viable organisms including fungi (Lepp 2005). This information leads to a probable correlation between the presence of humans, and gulls associated with the waste produced by humans, and the fungi found in the environment surrounding both animals. This fungal presence is worthy of concern, since individuals with weakened immune systems are susceptible to fungal infections, which range from superficial to systemic mycoses. Moace and his colleagues demonstrated that almost all AIDS patients within their study had a fungal infection of some kind. Species found in these infections included *C. neoformans* and multiple biotypes of *Candida albicans* (Rees and Dixon 1981). Not only were these species present within these individuals but were also they were also systemic in nature, spreading to areas not within the initial zone of infection. Therefore it is hypothesized that there are pathogenic yeast-like fungi present in the guano of seagulls, that these fungi are deposited in areas around Myrtle Beach, and that they may present a potential health risk to beach-goers.

Methods and Materials

Sampling: Seagull guano samples were collected from nonporous surfaces at public

beach accesses at 26th Avenue N, 38th Avenue N, 45th Avenue N, 53rd Avenue N, 64th Avenue N,

and 76th Avenue N all within the city limits of Myrtle Beach, South Carolina. Each sample was

collected in a test tube, placed in a sealable plastic bag and labeled. These samples were then

refrigerated until they were processed in the laboratory. The samples were weighed so that

fungal concentrations could be presented as colony forming units per gram of feces.

Isolation: Samples were dissolved in 4 ml of sterile distilled water and four ten-fold

serial dilutions were made for each of the samples. Each dilution was then plated in quadrants

on Rose Bengal Agar. The plates were incubated at 37°C for one week. Individual colonies

were then selected, based on morphology (color, size, and shape), and placed into 10 ml of

Sabouraud Dextrose Broth (SBD). They were then incubated at 37°C on a slow-moving shaker.

After one week the cultures were checked and 10 ml of Sabouraud Dextrose Broth was added to

each culture. They were allowed to grow for an additional week to provide more cellular growth

using the initial temperature and shaker settings.

Extraction of Genomic DNA: Ten milliliters of each culture was then centrifuged for

five minutes at 1200 x g at room temperature. The supernatant was poured off and the pellet was

resuspended in 0.5 ml of water. This suspension was then transferred to a microcentrifuge tube

and spun for five seconds at room temperature. The supernatant was then poured off and the

pellet was resuspended in 200 μl of breaking buffer (2% Triton X-100, 1% sodium dodecyl

sulfate, 100mM NaCl, 10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0), 0.3 g of glass beads was

added to each sample along with 200 μl of phenol/chloroform/isoamyl alcohol (50:48:2). Each

sample was vortexed for 30 seconds followed by 30 seconds on ice and then repeated for three minutes. Two hundred microliters of Tris-EDTA (TE) buffer, which was made with 10mM Tris buffer at a pH of 7.5 and 1mM EDTA, was then added to each sample and vortexed briefly. The samples were then microcentrifuged at 16,100 x g for five minutes and the aqueous layer was then transferred to a new clean microcentrifuge tube. One milliliter of cold 100% ethanol was added to each tube, mixed and left on ice for twenty minutes. Each tube was then microcentrifuged for three minutes at high speed at room temperature. The supernatant was then removed and the cell pellet was resuspended in 0.4 ml of TE Buffer. To degrade all RNA fragments 30 µl of 1 mg/ml of DNase-free RNase A was added to each tube. These tubes were then mixed and placed in an incubator at 37°C for five minutes. Ten microliters of 4 M ammonium acetate and 1 ml of cold 100% ethanol was added to each tube and mixed. The samples were then microcentrifuged for three minutes at high speed (16,100 x g) at room temperature. The supernatant was then removed and the pellet was dried using a vacuum microcentrifuge for fifteen minutes. The isolated genomic DNA was then resuspended in 100 µl of TE buffer.

Amplification with Universal Primers: For each sample the following PCR reaction mixture was made: 10 µl of 2x Promega PCR Master Mix, 1 µl of the SR6R (5'-AAGTAAAGT-CGTAACAAGG-3') primer, 1 µl of the LR1 (5'-GGTTGGTTT-CTTTTCCT-3') primer (these primers amplify the ITS-5.8S-ITS2 region of ribosomal DNA), 1 µl of template DNA, and 7 µl of nuclease-free water. These reactions were prepared on ice and then placed in a thermocycler (Bio-Rad, Hercules, CA) with the following settings, 94°C for two minutes, 94°C for one minute, 50°C for 45 seconds, 72°C for one minute for one cycle, 94°C for one minute, 50°C for 45 seconds, 72°C for one minute for 29 cycle, followed by 72°C for seven minutes and cooled to

4°C until they were removed. The PCR products were checked using agarose gel electrophoresis. A 0.8% agarose gel was prepared, 5 μl of PCR product was added to the gel along with 1 μl of 6x dye. The gel was run at 100V in a TE buffer under the same concentration as mentioned above. To determine the concentration of each PCR product, a PCR DNA ladder (Gen Script, Piscataway, NJ) with a known concentration was used and run along side the PCR products in the gel. The 500 bp band of the ladder used is at least three times more intense than the brightest of the other bands with a known concentration of 20 ng/μl, thus it was determined that in 1:6 dilution of the ladder 4 ng/μl of the 500bp band was present. This information was used to determine the concentration of PCR products.

Sequencing PCR Products: The PCR products were cleaned in order to proceed with the sequencing reactions using the ExoSapit PCR clean-up Kit (GE Healthcare LifeSciences, Buckinghamshire, UK). Two microliters of each PCR product was added to 6 μl of nuclease-free water and 3 μl of ExoSAPit. These tubes were incubated for fifteen minutes at 37°C and then for another 15 minutes at 80°C. Stock solutions of the cycle sequencing solution were made using Big Dye 1.1 (applied Biosystems Inc., Foster City, CA) which consisted of 14 μl of Big Dye, 7 μl of 5x Buffer, 2.31 μl of 10 μM SR6R primer and 25.9 μl of nuclease-free water. Seven microliters of the sequencing mix stock solution was then added to 3 μl of PCR product. The samples were then run in a PCR machine for 60 seconds at 94°C followed by 25 cycles of 10 seconds at 94°C, 10 seconds at 50°C, and 4 minutes at 60°C. The PCR products were then isolated using Centri-Sep columns (Applied Biosystems Inc., Foster City, CA). Once these columns were rehydrated and washed following the manufacturers instructions by adding nuclease free water to the gel and allowing it to settle for 30 minutes, 20 μl of each of the sequencing reaction mixture was added to the top of the gel, directly onto the center of the gel

bed. The columns were placed into a sample collection tubes and spun for two minutes at 750 x

g. The purified sample was then collected in the bottom of a sample collection tube. The

samples were then dried in a vacufuge (Eppendorf, Westbury, NY) for 45 minutes. In order to

sequence the products, 20 μl of 100% deionized formamide was added to each tube in order to

reconstitute the dried DNA. These samples were transferred to sequencing tubes and incubated

at 95°C for three minutes. Finally the samples are loaded into the sequencer (Applied

Biosystems Inc, Foster City, CA) and the sequences of the amplified regions of DNA were

obtained.

Results

 Sample Enumeration

  The weight of each of the samples is represented in Table 1. These data were

then used after the colonies were grown to determine the number of organisms per gram of

seagull feces. During the counting process the colonies were differentiated based upon size,

appearance, and color. An example of the different types of colonies present can be seen in

Figure 1, where all organisms have a pink tint, due to the pigment of the rose agar. Even without

the ability to differentiate between colors of organism, one may distinguish filamentous (arrow

A) and non-filamentous, yeast-like organisms (arrow B). Differences in the intensity of the pink

color may also indicate a difference in species, as darker fungi may contribute more pigment to

the color of the colony. Figure 2, shows fungal colonies isolated from beach sand in another

study in this lab, and makes it clear that one can distinguish differing colony morphologies on

solid media (arrow A versus arrow B). Colony forming units (CFUs) per gram of feces was then

recorded in Table 1. Samples 3 and 6 had too much growth to determine the number of colonies

formed. Sample 4 did not yield any growth at all. The remaining colonies yielded from 30 to

2,000 colony-forming units per gram of feces. This wide range shows a considerable degree of

ariation in number of fungi present in different samples. Some plates contained anywhere from

one large colony that overgrew the plate at a certain concentration, to thirty colonies. From all of

the plates ten distinct colonies were selected for identification. Instead of using microscopy to

determine the species of fungi, it was decided to use a DNA-sequenced based approach for

identification. This required the extraction of DNA from the colonies present.

DNA Extraction and PCR amplification

Ten colonies were selected to be cultured on SBD. Following culturing eight of the ten

samples yielded chromosomal DNA that could be used in PCR amplification. These samples

were labeled 1A, 1B, 1C, 2A, 4A, 4B, 5A, and 7A. The number in the ID corresponds with the

location the colony originated from (one of the beach samplings), followed by a letter that is

based upon the order in which it was isolated from a common plate. No successful

amplifications were obtained from the first PCR attempt. However, after the second PCR was

performed, PCR products were obtained for six of the extracted samples, as shown in Figure 3.

There was amplified DNA present in the seventh lane but it was unusable due to multiple PCR

products. Of all of the PCR products it is observed that the first six may be usable. All of the

DNA size is around 500 bp, but each sample differs slightly from this size. This may indicate

that the presence of several distinct species, as the size difference in these bands is likely to arise

from species-specific heterogeneity in interspatial sequence length.

Once the successful PCR products were obtained, there was break in between the first

and second semesters and the products were not further processed for sequencing. Upon return

from the break the PCR product was rechecked using gel electrophoresis and it was determined that the DNA was too badly sheared to be used for sequencing.

A third PCR was then performed to obtain new products. This round was successful and yielded five successful amplifications out of eight original samples, as determined by agarose gel electrophoresis. These samples were 1B, 1C, 2A, 4A, and 5A. Using this confirmatory gel, the concentrations of the PCR products were determined using known concentrations of the DNA ladder that was placed in the gel. The concentrations of the PCR products were as follows: 1B 16 ng/μl, 1C 20 ng/μl, 2A 12 ng/μl, 4A 20 ng/μl, and 5A 12 ng/μl. Once the concentration of the PCR product was determined the samples were then prepared for sequencing as indicated in the Materials and Methods and sequenced. There were no successful sequences obtained from the five samples used. The PCR product, from above, was then rechecked using gel electrophoresis and it was determined that only sample 1B, 1C, and 4A could be used, the other two samples (2A and 5A), both contained double bands which are unfavorable for sequencing. These three samples were then prepared and sequencing reactions were performed using the PCR products from the five samples as template DNA. Two positive controls were also used to ensure sequencing accuracy. The controls used were pGEM vector, with a known sequence, and the same primer at two different concentrations. The sequencing reactions were the analyzed using a sequencer. Sequence was not obtained from either samples or positive controls.

Discussion

The purpose of this study was to extract and identify the potentially pathogenic fungi found in seagull guano, by initially growing and isolating species based upon morphology, and then identification through molecular techniques.

Initially the guano samples were plated on Bengal Rose Agar. There were several different colony types identified on the plates that were grown. Eight colonies total were isolated based upon morphology from the seven sample plates collected. These samples successfully grew at 37°C, which indicates that they may be potential pathogens, since 37°C is the average human body temperature. If organisms cannot grow in this temperature range then it is likely that they will not be pathogenic due to their inability to grow within the human body. Based upon the colony forming units per gram, these concentrations range from 30 to 2000 organisms per gram. Based upon previously obtained information it is known that there are approximately 3×10^5 viable organisms present in the fecal matter of seagulls, the data from this study indicates that only 0.667% of these viable organisms are potentially pathogenic fungi. While this is a wide range for concentrations of fungi, if compared to other viable organisms, these values are relatively low. It is possible that some of the fungal organisms present in the fecal matter did not grow because the study was meant to isolate potentially pathogenic species. This restricts the growth to only those organisms that can survive at 37°C, the average human body temperature. It is also possible that the growth media used did not support the growth of all possible fungal organisms, as some fungi require special media. While these counts are relatively low, if they are introduced into a human microenvironment, even in the smallest amounts, whether by inhalation or through an abrasion, an infection may readily occur.

The first PCR amplification did not work because the thermocycler was incorrectly programmed. The second PCR product became unusable due to being frozen in a frost-free freezer for an extended period of time during which the PCR product becomes increasingly unstable and appears to "break down" when viewed on a DNA gel. This shearing prevents sequencing reactions from occurring properly because the DNA is not a solid piece but rather in

multiple fragments. Thus each separate fragment gets sequenced and the computer reading the sequence cannot place the fragments in the correct order because it is unknown.

The sequencing reactions did not work properly in either of the two attempts. In the first attempt it is suspected that the primer used was degraded in some way, or that the template DNA being used was somehow compromised. This led to running the third PCR product on another gel in order to check its integrity. This gel showed that two of the five PCR products contained multiple bands. Multiple bands create problems in sequencing since the sequencer is unable to determine the difference between the two bands, for example if two fragments of the same length came through the detector with the same size then the computer would incorrectly align them either side by side or on top of each other. In reality these two bands could have been sequenced from two different fragments, thus leading to wrong sequence being produced.

As a result of the multiple bands problem it was decided to set aside these two products and use the other three remaining intact products to try the sequencing reaction again. Along with this second round of sequencing reactions two controls were added. Both were PGEM plasmid but differed in the concentrations of the primers that were used. Unfortunately for this experiment none of the sequencing reactions worked. It is fortunate for this experiment, however, that the known controls did not work. This error can then most likely be attributed to a faulty reagent used in the sequencing reaction. The Big Dye 1.1 sequencing reagent was used from another lab. This stock had most likely been frozen and thawed several times over the course of its shelf life, thus potentially reducing its functionality. Another cause for this error could also have been the failure to add a reagent in the mixture, or rather the incorrect amount of reagent that was common in all reaction tubes. The reactions would have to be performed again and different reagents could be used at different amounts to confirm either of these errors.

The second possibility for the lack of success with the sequencing reactions could also have been with the thermocycler used. It is possible that the denaturation temperature was not set high enough to denature either the primer or the control DNA sequence. This would result in the primer being unable to anneal to its matched site on the DNA, and would prevent any polymerase reactions from occurring.

In future studies or continuation of this particular experiment it will be necessary to perform trouble-shooting steps to determine the exact cause for the lack of sequence being produced. It would also be beneficial to determine the source of the multiple bands of DNA present in some of the PCR products. Either the cause of this could be eliminated using different primers or perhaps an adjustment in the thermocycler settings. These bands could also be extracted individually and sequenced separately. Finally the experiment could be expanded to cover a much larger sampling range so that when samples do not yield successful products in different steps, there will be more to work with, instead of being reduced to three samples, as was the case in this study.

While the failure of the sequencing reactions precluded making definitive identifications of the species present in these samples, the study did support other findings. There were definitely potentially pathogenic fungi present in the samples obtained from recreational beaches. This study also provides the basis for further research in Dr. Aguirre's lab. The protocols may be used as the template for these future studies to isolate and identify pathogenic fungi in other samples, whether other bird fecal samples or another source. It is also highly apparent that when performing sequencing reactions that control DNA templates should be used as well as fresh reagents. Overall it is apparent that seagull guano acts as a carrier for fungi whose presence could impact the health of beach-goers. The relative significance of this source, as well as the

other potential sources, may lend suggestions for controlling the presence of potentially

pathogenic fungi on recreational beaches to public health officials.

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Finally I should also thank Molly French who provided me with keys to doors when I was locked out. And to Ashley Moore who helped me in the lab with this project, and kept me from getting too bored while waiting for samples to be heated, thawed, or dried.

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Figures and Tables

Table 1

Sample	1	2	3	4	5	6	7
Weight (g)	0.66	0.70	0.70	0.67	0.79	0.67	0.67
Concentration (CFU/g)	2.0×10^3	5.0×10^2	Too Overgrown	No Growth	9.0×10^2	Too Overgrown	3.0×10

This table represents the concentration of organisms per gram of feces. As noted in the table both samples 3 and 6 had too much growth to determine the number of colonies formed. In addition to this, sample 4 yielded no growth at all. The remaining colonies per gram range from 30 to 2,000.

Figure 1:
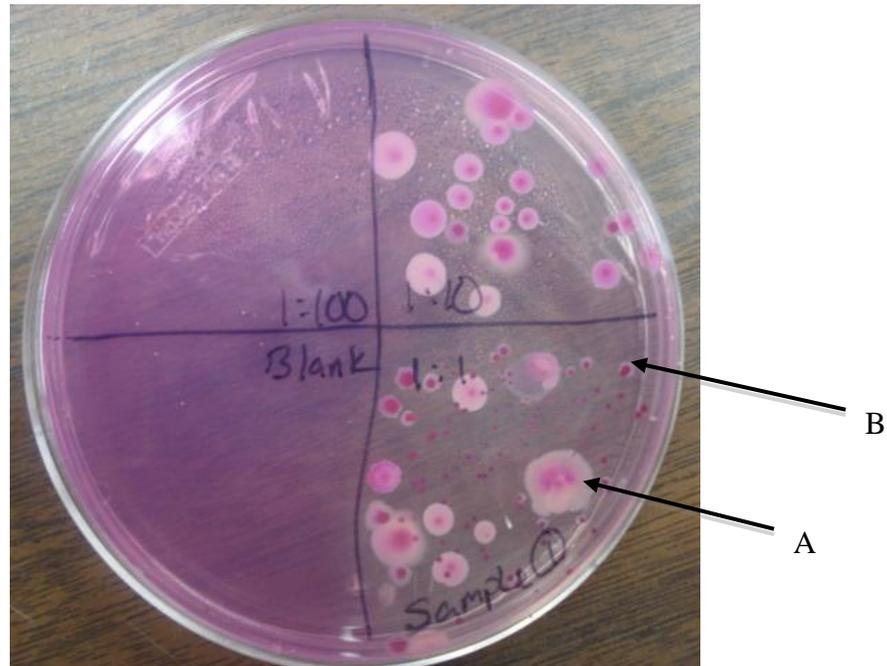
This figure shows the different types of colonies that formed during growth. All organisms have a pinkish hue due to the Bengal Rose Agar pigment. Even without the ability to differentiate between colors of organism, one may distinguish filamentous (arrow A) and non-filamentous, yeast-like organisms (arrow B). Differences in the intensity of the pink color may also indicate a difference in species, as darker fungi may contribute more pigment to the color of the colony. Differences in the size of the colonies may reflect species differences in doubling time.