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Charakterystyka patogenności mikroorganizmów bytujących w organizmie człowieka oraz zwierząt towarzyszących na terenie Polski wschodniej– aspekty epidemiologiczne, molekularne i środowiskowe

Characteristics of pathogenicity of microorganisms living in the human body and companion animals in eastern Poland – epidemiological, molecular and environmental aspects

Praca doktorska
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Streszczenie

Charakterystyka patogenności mikroorganizmów bytujących w organizmie człowieka oraz zwierząt towarzyszących na terenie Polski wschodniej– aspekty epidemiologiczne, molekularne i środowiskowe

Infekcje odkleszczowe, jak i zakażenia grzybicze stanowią rosnące wyzwanie epidemiologiczne, zwłaszcza w kontekście ich zmieniającej się częstości występowania oraz różnic regionalnych. Celem niniejszej pracy było zintegrowane scharakteryzowanie mikroorganizmów o potencjale patogennym bytujących w organizmach ludzi oraz zwierząt towarzyszących (głównie psów) na terenie Polski wschodniej, z uwzględnieniem ich znaczenia epidemiologicznego, molekularnego i środowiskowego. Badania koncentrowały się na dwóch głównych grupach patogenów: drobnoustrojach przenoszonych przez kleszcze, takich jak *Borrelia burgdorferi* i *Anaplasma phagocytophilum* oraz grzybach z rodzaju *Candida*, zasiedlających błony śluzowe jamy ustnej człowieka. Zwierzęta towarzyszące, takie jak psy i koty, stanowią istotny element życia społecznego ludzi, zwłaszcza w warunkach miejskich oraz podmiejskich. Jednocześnie pełnią rolę potencjalnych rezerwuarów lub wektorów licznych patogenów o znaczeniu zoonotycznym. W dynamicznie zmieniającym się środowisku pod wpływem zmian klimatycznych, urbanizacji i migracji zwierząt wzrasta ryzyko występowania i transmisji chorób odzwierzęcych, w tym tych przenoszonych przez kleszcze (tick-borne diseases) oraz związanych z mikroflorą endogenną. Wśród najczęściej spotykanych patogenów przenoszonych przez kleszcze znajdują się bakterie z rodzaju *Borrelia* (czynnik etiologiczny boreliozy) oraz *Anaplasma phagocytophilum*, powodująca anaplazmozę granulocytarną u ludzi i zwierząt. Choroby te stanowią narastające zagrożenie nie tylko dla zdrowia publicznego, ale również dla medycyny weterynaryjnej i ochrony środowiska. Udział zwierząt towarzyszących w cyklu biologicznym tych patogenów oraz rola lokalnych populacji kleszczy w ich transmisji wymagają stałego monitorowania i analizy molekularnej. Równolegle, coraz większe znaczenie przypisuje się grzybom drożdżopodobnym z rodzaju *Candida*, które w warunkach prawidłowego funkcjonowania organizmu człowieka stanowią element fizjologicznej mikrobioty błon śluzowych, głównie jamy ustnej, przewodu pokarmowego oraz dróg moczowo-płciowych. Jednakże w sytuacjach zaburzenia homeostazy m.in. w wyniku antybiotykoterapii, osłabienia odporności, stresu oksydacyjnego czy powstawania biofilmów *Candida* może przekształcać się w patogen oportunistyczny, zdolny do wywołania powierzchownych oraz układowych zakażeń kandydozowych. W badaniach nad zjadliwością *Candida albicans* coraz większy nacisk kładzie się na właściwości nanomechaniczne komórek takie jak sztywność błony komórkowej czy zdolność do adhezji, które mogą odgrywać istotną rolę w kolonizacji gospodarza i formowaniu biofilmów. Wykazano także, że formy planktoniczne i biofilmowe wykazują różne cechy strukturalne i odpornościowe. Zróżnicowanie genotypowe w obrębie izolowanych szczepów wpływa zarówno na ich fenotyp, jak i zdolność adaptacji do środowiska gospodarza. Te właściwości fizyko-chemiczne i genetyczne *Candida* stanowią nowe pole badań nad mechanizmami patogenezy i potencjalnymi celami terapeutycznymi. Zrozumienie lokalnego występowania i zmienności patogenów na poziomie molekularnym, ekologicznym i fenotypowym jest kluczowe dla skutecznej prewencji i leczenia zakażeń o znaczeniu zdrowia publicznego.

Słowa kluczowe: *Candida*, biofilm, kleszcze, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, zoonozy, infekcje grzybicze

Abstract

Characterization of pathogenicity of microorganisms inhabiting the human body and companion animals in eastern Poland – epidemiological, molecular and environmental aspects

Tick-borne infections and fungal infections constitute a growing epidemiological challenge, especially in the context of their changing frequency of occurrence and regional differences. The aim of this study was to provide an integrated characterization of microorganisms with pathogenic potential inhabiting the bodies of humans and companion animals (mainly dogs) in eastern Poland, taking into account their epidemiological, molecular and environmental significance. The studies focused on two main groups of pathogens: microorganisms transmitted by ticks, such as *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, and fungi of the *Candida* genus, inhabiting the mucous membranes of the human oral cavity. Companion animals, such as dogs and cats, are an important element of human social life, especially in urban and suburban conditions. At the same time, they act as potential reservoirs or vectors of numerous pathogens of zoonotic importance. In a dynamically changing environment, due to climate change, urbanization, and animal migration, the risk of occurrence and transmission of zoonotic diseases, including those transmitted by ticks (tick-borne diseases) and those related to endogenous microflora, is increasing. The most common pathogens transmitted by ticks include bacteria of the genus *Borrelia* (the etiological factor of Lyme disease) and *Anaplasma phagocytophilum*, which causes granulocytic anaplasmosis in humans and animals. These diseases pose a growing threat not only to public health, but also to veterinary medicine and environmental protection. The participation of companion animals in the biological cycle of these pathogens and the role of local tick populations in their transmission require constant monitoring and molecular analysis. At the same time, increasing importance is attributed to yeast-like fungi of the genus *Candida*, which, under conditions of normal functioning of the human body, are an element of the physiological microbiota of the mucous membranes, mainly the oral cavity, the gastrointestinal tract, and the urinary-genital tract. However, in situations of disruption of homeostasis, e.g. as a result of antibiotic therapy, weakened immunity, oxidative stress or the formation of biofilms, *Candida* can transform into an opportunistic pathogen capable of causing superficial and systemic candidiasis infections. In studies on the virulence of *Candida albicans*, increasing emphasis is being placed on the nanomechanical properties of cells, such as the stiffness of the cell membrane or the ability to adhere, which may play an important role in host colonization and the formation of biofilms. It has also been shown that planktonic and biofilm forms exhibit different structural and immune features. Genotypic diversity within isolated strains affects both their phenotype and their ability to adapt to the host environment. These physicochemical and genetic properties of *Candida* constitute a new field of research on the mechanisms of pathogenesis and potential therapeutic targets. Understanding the local occurrence and variability of pathogens at the molecular, ecological and phenotypic level is crucial for effective prevention and treatment of infections of public health importance.

Keywords: *Candida*, biofilm, ticks, *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, zoonoses, fungal infections

Spis treści

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1. Wstęp

Współczesna mikrobiologia zwierząt i ludzi coraz częściej koncentruje się na złożonych relacjach zachodzących pomiędzy gospodarzem a kolonizującymi go mikroorganizmami. W szczególności coraz większe znaczenie przypisuje się mikroflorze oportunistycznej oraz patogenom przenoszonym przez wektory, które nierzadko występują jako część fizjologicznego mikrobiomu i w sprzyjających warunkach mogą prowadzić do poważnych infekcji, zarówno miejscowych, jak również układowych. Dynamiczne zmiany środowiskowe, postępująca urbanizacja, zmiany klimatyczne oraz intensyfikacja kontaktu człowieka ze zwierzętami domowymi i dzikimi sprawiają, że liczba zakażeń o złożonej etiologii mikrobiologicznej systematycznie wzrasta. W tym kontekście szczególnego znaczenia nabierają drożdżaki z rodzaju *Candida* oraz kleszcze, jako wektory groźnych patogenów bakteryjnych i pierwotniaczych [Blaszkowska i wsp., 2023; Caban i wsp., 2024].

Drożdżaki, a zwłaszcza gatunki należące do kompleksu *Candida albicans*, są powszechnie występującymi organizmami zasiedlającymi błony śluzowe przewodu pokarmowego, jamy ustnej, pochwy oraz skóry człowieka i zwierząt. W warunkach prawidłowych funkcjonują one jako komensale, odgrywając rolę w utrzymaniu równowagi mikrobiologicznej organizmu. Niemniej jednak, zaburzenia odporności, przewlekła antybiotykoterapia, obecność cewników, protez lub innych ciał obcych, niedobory żywieniowe, modyfikacje w diecie, a także zmiany w składzie mikrobioty mogą doprowadzić do ich przekształcenia w formy patogenne [Pieczyńska i wsp. 2020]. W takim stanie drożdżaki stają się zdolne do aktywnego namnażania, inwazji tkanek oraz wywoływania różnorodnych objawów klinicznych – od powierzchownych infekcji skóry i błon śluzowych po ciężkie, potencjalnie śmiertelne zakażenia uogólnione, znane jako kandydozy inwazyjne [Cafarchia i wsp., 2024].

Właściwości patogenne *Candida* spp. są ściśle powiązane z ich zdolnością do przylegania do powierzchni biologicznych i sztucznych oraz tworzenia biofilmów. Biofilmy te składają się z gęstej sieci komórek grzybiczych otoczonych macierzą zewnątrzkomórkową, która chroni je przed działaniem leków przeciwgrzybiczych oraz układu immunologicznego gospodarza. Organizmy te potrafią również przekształcać się morfologicznie, przechodząc z formy jednokomórkowej drożdżowej do formy strzępkowej lub pseudostrzępkowej, co stanowi dodatkowy czynnik wirulencji. Co więcej, niektóre szczepy *Candida* wykazują oporność na popularne leki przeciwgrzybicze, takie jak flukonazol czy amfoterycyna B utrudniając leczenie i zwiększając ryzyko nawrotów infekcji. Obserwuje się także coraz większe znaczenie tzw. gatunków nie-albicans, takich jak *Nakaseomyces glabratus* (dawniej *Candida glabrata)*, *Pichia*

kudriavzevii (dawniej *C. krusei*), *C. parapsilosis* czy *C. auris*, które wykazują odmienne właściwości biologiczne i często większą oporność na leczenie oraz trudności w diagnostyce [Pristov i wsp., 2019; Borman i wsp., 2023].

Równolegle z problemem zakażeń grzybiczych, znaczące zagrożenie stanowią patogeny przenoszone przez kleszcze będące wektorami licznych drobnoustrojów chorobotwórczych. Na terenie Polski Wschodniej dominującymi gatunkami kleszczy są *Ixodes ricinus* oraz *Dermacentor reticulatus*, które ze względu na sprzyjające warunki środowiskowe występują nie tylko w lasach i na terenach wiejskich, lecz coraz częściej również w parkach miejskich, ogródkach działkowych oraz bezpośrednim sąsiedztwie ludzkich osiedli. Ekspansja terytorialna tych pasożytów w ostatnich latach związana jest z ociepleniem klimatu, wzrostem liczby gospodarzy pośrednich (np. gryzoni) oraz zwiększonym ruchem zwierząt towarzyszących pomiędzy obszarami miejskimi i wiejskimi [Mierzejewska wsp., 2022; Stańczak i wsp., 2020]. Kleszcze przenoszą szereg drobnoustrojów odpowiedzialnych za poważne choroby ludzi i zwierząt. Wśród najważniejszych patogenów znajduje się *Borrelia burgdorferi* sensu lato, wywołująca boreliozę z Lyme – najczęstszą chorobę odkleszczową w Europie. Inne istotne patogeny obejmują *Anaplasma phagocytophilum*, czynnik etiologiczny anaplazmozy granulocytarnej, oraz *Babesia* spp., wywołujące babeszjozę – chorobę przypominającą malarię. Przebieg tych zakażeń może być ostry lub przewlekły, często nieswoisty, co znacząco utrudnia diagnostykę kliniczną. W przypadku zwierząt towarzyszących, takich jak psy i koty, objawy mogą obejmować gorączkę, niedokrwistość, apatię, utratę masy ciała oraz zaburzenia neurologiczne, co ma poważne implikacje nie tylko zdrowotne, lecz także zoonotyczne [Rizzoli i wsp., 2014].

Istotnym problemem w kontekście badań epidemiologicznych i molekularnych jest współwystępowanie wielu patogenów w obrębie jednego kleszcza oraz możliwość jednoczesnego zakażenia kilku organizmów gospodarzy. Koinfekcje takie mogą prowadzić do interakcji patogen-patogen oraz patogen-gospodarz o nieprzewidywalnych skutkach klinicznych. Współczesne badania wykazują, że kleszcze są nie tylko wektorami, ale także swoistymi „biologicznymi magazynami" patogenów, w których może dochodzić do przemian genetycznych, selekcji opornych szczepów oraz adaptacji do nowych gospodarzy [Mierzejwska i wsp., 2022].

Zarówno grzyby drożdżopodobne, jak i patogeny przenoszone przez kleszcze należy rozpatrywać w szerszym kontekście ekologicznym i zdrowotnym, integrującym zdrowie człowieka, zdrowie zwierząt oraz stan środowiska. Szczególne znaczenie mają w tym kontekście zwierzęta towarzyszące, które ze względu na bliski kontakt z człowiekiem mogą

stanowić zarówno wskaźniki środowiskowe obecności patogenów, jak i rezerwuary infekcji zoonotycznych [Mierzejewska i wsp., 2022].

Obecność mikroorganizmów oportunistycznych oraz wektorowo przenoszonych patogenów w organizmach ludzi i zwierząt domowych wskazuje na potrzebę ich kompleksowego badania w ujęciu epidemiologicznym, molekularnym i środowiskowym. Zrozumienie mechanizmów patogenności, zmienności genetycznej, interakcji mikroorganizmów oraz ich wpływu na zdrowie gospodarza jest niezbędne dla opracowania skutecznych strategii prewencji, diagnostyki i leczenia chorób zakaźnych. Niniejsza praca doktorska stanowi próbę integracji danych z zakresu mikrobiologii, parazytologii oraz ekologii zdrowia w celu pogłębienia wiedzy na temat zagrożeń mikrobiologicznych występujących na styku człowieka, zwierząt towarzyszących i środowiska naturalnego na obszarze Polski Wschodniej.

2. Hipotezy badawcze, cel i zakres pracy

Postawiono następujące hipotezy badawcze:

1. Izolaty drożdżaków z rodzaju *Candida*, pochodzące z jamy ustnej zdrowych osób zamieszkujących region Polski wschodniej, wykazują istotne różnice w profilu genotypowym oraz fenotypowym (w tym zdolności do tworzenia biofilmu, filamentacji i wzrostu w podwyższonej temperaturze) w porównaniu z danymi referencyjnymi dla populacji spoza tego regionu.
2. Parametry nanomechaniczne ściany komórkowej szczepów *Candida albicans* (m.in. sztywność, adhezja powierzchniowa), oceniane za pomocą mikroskopii sił atomowych (AFM), korelują istotnie z ich właściwościami patogenetycznymi, takimi jak zdolność do tworzenia biofilmu i filamentacji.
3. Na obszarze Polski wschodniej odnotowuje się wyższe rozpowszechnienie patogenów przenoszonych przez kleszcze (*Borrelia burgdorferi* sensu lato oraz *Anaplasma phagocytophilum*) u psów domowych niż w populacjach referencyjnych z innych regionów kraju, co może wskazywać na regionalne zróżnicowanie ryzyka epidemiologicznego.
4. Gatunki kleszczy występujące u psów we wschodniej Polsce stanowią istotny rezerwuar i wektor *Anaplasma phagocytophilum*, niezależnie od płci kleszcza oraz statusu socjalnego psa (domowy vs bezdomny), natomiast jego rola w transmisji *Borrelia burgdorferi* sensu lato jest ograniczona.

Celem głównym pracy jest kompleksowa charakterystyka patogenności wybranych mikroorganizmów kolonizujących ludzi oraz zwierzęta towarzyszące (psy) na terenie Polski wschodniej w odniesieniu do warunków środowiskowych i kontekstu epidemiologicznego. Praca ma na celu określenie, w jaki sposób mikroorganizmy oportunistyczne oraz patogeny przenoszone przez wektory mogą stanowić zagrożenie zdrowotne zarówno dla ludzi, jak i zwierząt domowych oraz zbadanie wpływu lokalnych uwarunkowań środowiskowych na występowanie i właściwości tych mikroorganizmów, co pozwoli na lepsze zrozumienie mechanizmów ich adaptacji i patogenności w kontekście regionu Polski wschodniej.

Realizacja celu głównego została osiągnięta poprzez:

- izolację i identyfikację wybranych mikroorganizmów z materiału biologicznego ludzi i zwierząt,

- ocenę ich cech fenotypowych i molekularnych (m.in. biofilm, filamentacja, analiza DNA),
- zastosowanie technik mikroskopii sił atomowych do oceny właściwości fizykochemicznych,
- analizę obecności patogenów wektorowych u psów z różnych środowisk,
- integrację uzyskanych danych z analizami środowiskowymi i epidemiologicznymi.

3. Materiały i metody

Publikacja 1 (P1) Ocena zróżnicowania gatunkowego szczepów *Candida* kolonizujących jamę ustną osób zdrowych oraz określenie relacji genotyp – fenotyp u *Candida albicans* (Andrzejuk i wsp., 2025).

Grupa badawcza składała się z 500 uczestników (288 kobiet, 212 mężczyzn) w wieku 18–90 lat (średni wiek 39 lat; σ = 16,79). Wymazy z jamy ustnej pobrano od zdrowych ochotników bez objawów infekcji lub chorób współistniejących. Próbki pobrano za pomocą jałowych wymazówek z medium transportowym, pocierając wewnętrzną stronę policzka przez 15 s z każdej strony. Hodowlę przeprowadzono na podłożu Sabouraud dekstroza (Argenta, Poznań, Polska) przez 24 h w temperaturze 35°C. Pozyskane szczepy bankowano (Mast Group, Reinfeld, Niemcy) i przechowywano w temperaturze − 20°C.

Metody fenotypowe obejmowały:

- hodowlę na podłożach chromogennych (CHROMagar, GRASO, Polska) temperaturze 35°C przez 48 godzin w celu wstępnej identyfikacji gatunku;
- analizę asymilacji cukrów z wykorzystaniem testu kolorymetrycznego AUXACOLOR 2 (Bio-Rad, Warszawa, Polska), gdzie inokulum (standard McFarland 1,5, 100 µL) przygotowano z 24-godzinnej hodowli i inkubowano w temperaturze 30°C;
- testy tolerancji temperaturowej, w których próbki inkubowano na podłożu stałym Sabouraud w temperaturze 42°C, a obserwacje wykonywano po 24, 48 i 72 godzinach;
- testy filamentacji – próbki dostosowano do standardu McFarlanda 2 z dodatkiem 0,5 ml surowicy ludzkiej, inkubowano w temperaturze 37°C przez około 2,5 godziny i prowadzono obserwacje pod mikroskopem (Nikon Eclipse 80i, Warszawa, Polska).

Analizy genotypowe uwzględniały:

- izolację genomowego DNA z 24-godzinnej hodowli o standardzie około 1,5 McFarlanda przy wykorzystaniu zestawu Genomic MiniKit (A&A Biotechnology, Gdańsk, Polska);
- analizę polimorfizmu długości fragmentów restrykcyjnych (RFLP – *restriction fragment length polymorphism*) regionu ITS charakteryzującego się stosunkowo konserwatywnymi sekwencjami oraz wysoką zmiennością z trawieniem restrykcyjnym

przy użyciu enzymu restrykcyjnego MspI (10 U/µl) (Thermo Scientific, Warszawa, Polska);

- genotypowanie na podstawie sekwencji 25S rDNA.

Po weryfikacji szczepów *Candida albicans* i określeniu ich genotypów wykonano następujące analizy:

- badanie wrażliwości na leki przeciwgrzybicze – określając minimalne stężenie hamujące (MIC) anidula-funginy, amfoterycyny B, mykafunginy, posakonazolu, worykonazolu, itrakonazolu i flukonazolu za pomocą testu SensititreYeastOne Thermo Scientific, Warszawa, Polska;
- analizy tworzenia biofilmów z 48-godzinnej hodowli i inkubacji w 35°C anem) przy użyciu mikroskopu optycznego Nikon (model Eclipse 80i) wyposażonego w kamerę Nikon DS-Fi1c.

Publikacja 2 (P2) Ocena właściwości nanomechanicznych *Candida albicans* (Teodorowicz i wsp., 2023).

Materiał do badań stanowiły wymazy zebrane z wewnętrznej części policzka od osób zdrowych bez objawów infekcji jamy ustnej. Hodowle przeprowadzono na podłożu agarowym Sabouraud z gentamycyną i chloramfenikolem (Oxoid) w temperaturze 35°C przez 48 godzin.

W celu identyfikacji i weryfikacji otrzymanych szczepów przeprowadzono:

- analizę asymilacji cukrów z wykorzystaniem testu kolorymetrycznego AUXACOLOR 2 (Bio-Rad, Warszawa, Polska), inokulum (standard McFarland 1,5, 100 µL) z 24-godzinnej hodowli i inkubowanej w temperaturze 30°C;
- określenie minimalnego stężenia hamującego (MIC) przy użyciu pasków gradientowych MTSTM i podłoża agarowego RPMI (Liofilchem).

Analizy mikroskopowe:

- skaningowy mikroskop elektronowy SEM - Hitachi model TM 3000 wyposażony w spektroskopię rentgenowską z dyspersją energii (Oxford Instruments). Próbki z materiałem biologicznym napylono cienką (kilka nanometrów) warstwą miedzi o czystości 99,999% (Testbourne, Wielka Brytania). Warstwę Cu osadzono za pomocą

systemu rozpylania magnetronowego DC (nano PVD, Moorfield) w atmosferze czystego argonu (99,999%) przy ciśnieniu 1,8 . 10-2 mbar. Zastosowano obrazowanie elektronów wstecznie rozproszonych z napięciem przyspieszającym 15 kV;

- technika mikroskopii sił atomowych AFM - NTEGRA Spectra C (NT-MDT) obejmowała analizę próbek w cieczy w temp. 21°C, w celu zapewnienie komórkom warunków fizjologicznych. wykonywano w cieczy, w temperaturze pokojowej (21°C). Wszystkie obrazy uzyskano przy użyciu bardzo miękkich, trójkątnych wsporników Si3N4 zaprojektowanych do obrazowania próbek biologicznych (MSCT Bruker) o nominalnej stałej sprężystości k~0,01 N/m i częstotliwości rezonansowej fo~7 kHz.

Publikacja 3 (P3) Ocena występowania zakażeń *Borrelia burgdorferi* i *Anaplasma phagocytophilum* w kleszczach *Ixodes ricinus* pobranych od psów we wschodniej Polsce (Pańczuk i wsp., 2024)

Materiał do badań stanowiło 147 dorosłych kleszczy *Ixodes ricinus* (81 samic i 66 samców) zebranych z psów żywicieli lub nosicieli w północno – wschodnich regionach województwa lubelskiego. Gatunek kleszcza, płeć i stadium rozwojowe zostały zidentyfikowane zgodnie z kluczem opracowanym przez Nowak-Chmurę. Kleszcze do czasu analiz przechowywano pojedynczo w probówkach Eppendorfa w 70% etanolu w temperaturze 6°C.

Analizy molekularne wykonano przy użyciu testu AmpliSens TBEV, *B.burgdorferi* s.l., *A.phagocytophilum*, *E.chaffeensis*/*E.muris*-FRT (Ecoli Dx, Bratysława, Republika Słowacka) do swoistego wykrywania fragmentu genu 16S rybosomalnego RNA w *B. burgdorferi* s.l. i fragmentu genu białka powierzchniowego merozoitu 2 w *A. phagocytophilum*.
Badanie obejmowało etapy:

- izolacji materiału genetycznego – z wykorzystaniem zestawu RIBO prep
- reakcji odwrotnej transkrypcji w celu syntezy komplementarnego DNA (cDNA) przy użyciu zestawu REVERTA-L
- reakcji PCR w czasie rzeczywistym (TBEV, B. burgdorferi s.l., A. phagocytophilum,
- Ehrlichia chaffeensis/E. muris-FRT PCR kit).

Publikacja 4 (P4) Ocena występowania zakażeń *Borrelia burgdorferi* i *Anaplasma phagocytophilum* w kleszczach *Dermacentor reticulatus* pobranych od psów we wschodniej Polsce (Pańczuk i wsp., 2022).

Materiał do badań stanowiło 152 dorosłych kleszczy *Dermacentor reticuatus* (71 samic i 81 samców) zebranych od 55 psów żywicieli lub nosicieli w północno – wschodnich regionach województwa lubelskiego. Gatunek kleszcza, płeć i stadium rozwojowe zostały zidentyfikowane zgodnie z kluczem opracowanym przez Nowak-Chmurę. Kleszcze do czasu analiz przechowywano pojedynczo w probówkach Eppendorfa w 70% etanolu w temperaturze 6°C.

Analizy molekularne wykonano przy użyciu testu AmpliSens TBEV, *B.burgdorferi* s.l., *A.phagocytophilum*, *E.chaffeensis*/*E.muris*-FRT (Ecoli Dx, Bratysława, Republika Słowacka) do swoistego wykrywania fragmentu genu 16S rybosomalnego RNA w *B. burgdorferi* s.l. i fragmentu genu białka powierzchniowego merozoitu 2 w *A. phagocytophilum*.
Badanie obejmowało etapy:

- izolacji materiału genetycznego – z wykorzystaniem zestawu RIBO prep
- reakcji odwrotnej transkrypcji w celu syntezy komplementarnego DNA (cDNA) przy użyciu zestawu REVERTA-L
- reakcji PCR w czasie rzeczywistym (TBEV, *B. burgdorferi* s.l., *A. phagocytophilum*,
- *Ehrlichia chaffeensis*/*E. muris*-FRT PCR kit).

4. Omówienie głównych wyników badań

Publikacja 1 (P1) Ocena zróżnicowania gatunkowego szczepów *Candida* kolonizujących jamę ustną osób zdrowych oraz określenie relacji genotyp – fenotyp u *Candida albicans* (Andrzejuk i wsp., 2025).

Szczepy *Candida* spp. wykryto w próbkach z wymazów jamy ustnej u 130 z 500 uczestników, co wskazuje, że 26% osób było nosicielami grzybów. Spośród 130 próbek *Candida*, 77% (100/130) zidentyfikowano jako *C. albicans*, 12% (16/130) *C. dubliniensis*, 8% (10/130) *P. kudriavzevii*, a 3% (4/130) *N. glabrata*. W tym badaniu nie wyizolowano więcej niż jednego gatunku od jednej osoby. *C. albicans* jest najczęściej występującym gatunkiem w tym środowisku ze względu na cechy takie jak wysoka patogenność, wirulencja i zdolność adaptacji [Millsop i wsp., 2016].
Do dalszych analiz weryfikacji fenotypowej wybrano 100 próbek wstępnie określonych jako *C.albicans* a ich wyniki zestawiono w Tabeli 1.

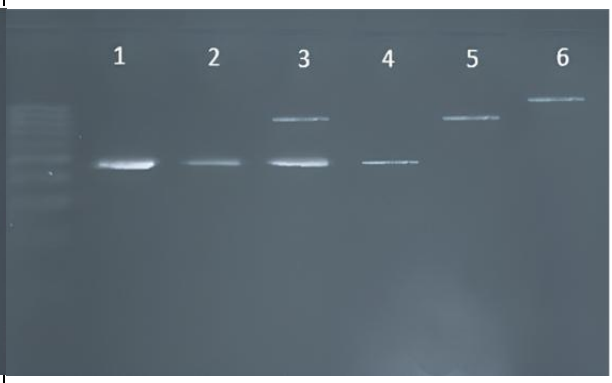
Tabela 1 Wyniki testów fenotypowych z wykorzystaniem testów AUXACOLOR na asymilację cukru, tolerancję temperatury, filamentację i lekowrażliwość Sensititre YeastOne.

	C. albicans	*C. dublinensis*
AUXACOLOR	97% (97/100)	3% (3/100)
Tolerancja temperaturowa	93% (93/100)	7% (7/100)
Testy filamentacyjne	97% (97/100)	3% (3/100)
Sensititre Yeast One	100% (100/100)	0% (0/100)

Identyfikację molekularną przeprowadzono na wszystkich wyizolowanych szczepach *Candida*. Pierwszy etap stanowiła reakcja PCR regionu ITS oraz trawienie enzymem restrykcyjnym MspI a otrzymane produkty charakteryzowały się rozmiarami od 510 do 870 pz. Wyniki elektroforezy dla *C. albicans* i *C. dubliniensis* były niemal identyczne. Nie jest to zaskakujące, biorąc pod uwagę ograniczenia związane z identyfikacją fenotypową. Wyniki te sugerują, że szczepy są silnie ze sobą powiązane [Zahir i wsp., 2012]. Drugi etap obejmował genotypowanie, szczepów zidentyfikowanych wstępnie jako *C. albicans* i wątpliwych *C.dublinensis*, oparte na sekwencji 25S rDNA, w którym pojedynczy produkt uzyskano dla genotypów A (450 pz.) w przypadku 70% i B (840 pz.) 17%, a podwójny produkt dla genotypu

C (450 pz. i 840 pz.) 9%. Stwierdzono również obecność *C.dublinesis* (1080 pz.) w 4%, co nie pokrywa się z wynikami uzyskanymi metodami fenotypowymi (Tabela 2).

Tabela 2. Wyniki analiz molekularnych produktów PCR regionu ITS1–5.8S-IT2 po trawieniu restrykcyjnym MspI oraz genotypowaniu opartym na sekwencji 25S rDNA.

region ITS1–5.8S-IT2	region 25S rDNA
	
ścieżka 1 kontrola negatywna; ścieżka 2 *C. albicans*; ścieżka 3 *C. dubliniensis*; ścieżka 4 *P. kudriavzevii*; ścieżka 5 *N. glabrata*; i ścieżka 6 *C. tropicalis*	ścieżki 1, 2 i 4 genotyp A *C. albicans*; ścieżka 3 genotyp C *C. albicans*; ścieżka 5 genotyp B *C. albicans*; ścieżka 6 *C. dubliniensis*

Analizę lekowrażliwości wykonano na szczepach zidentyfikowanych molekularnie jako *C.albicans*. Badane próbki określone genotypem B i C wykazywały wrażliwość na wszystkie leki przeciwgrzybicze. Próbki genotypu A określono jako wysoce oporne. W 57% (40/70) izolaty charakteryzowały się opornością na anidulafunginę, na posakonazol u 7,1% (5/70), na itrakonazol u 21,4% (15/70) i na worykonazol u 5,71% (4/70). Oporność powszechnego genotypu A *C. albicans* na leki przeciwgrzybicze azolowe i echinokandyny może przyczyniać się do niepowodzenia leczenia. Wynika to z faktu, że stanowią one podstawowe grupy leków stosowanych w potencjalnym zastosowaniu w terapii inwazyjnych chorób grzybiczych [Liu i wsp., 2008]. Dokładne wyniki wraz z wartościami MIC przedstawiono w Tabeli 3.

Tabela 3. Wartości MIC (mg/l) środków przeciwgrzybiczych wobec badanych szczepów *Candida albicans* (genotyp A, B lub C).

	Candida albicans species			
	Genotype A	*Genotype B*	*Genotype C*	
Number of isolates (n = 96)	70 (73%)	17 (18%)	9 (9%)	MIC breakpoint (mg/L)
Antifungal agents used for testing	MIC values (mg/L)			

FLUCONAZOLE	min–max	0.12–1	0.12–0.5	0.12–1	S ≤ 2
	SD	0.20	0.10	0.25	R > 4
	$\bar{x}$	0.27	0.29	0.37	
	CV	0.76	0.34	0.67	
POSACONAZOLE	min–max	0.15–0.8	0.015–0.3	0.008–0.3	
	SD	0.02	0.01	0	S ≤ 0.06
	$\bar{x}$	0.02	0.02	0.02	R > 0.06
	CV	0.94	0.65	0.28	
ITRACONAZOLE	min–max	0.015–0.5	0.015–0.06	0.015–0.06	
	SD	0.02	0.01	0.02	S ≤ 0.06
	$\bar{x}$	0.04	0.03	0.04	R > 0.06
	CV	0.44	0.38	0.42	
MICAFUNGIN	min–max	0.008–0.03	0.008–0.015	0.008–0.015	
	SD	0.01	0	0	S ≤ 0.016
	$\bar{x}$	0.01	0.01	0.01	R > 0.016
	CV	0.51	0.29	0.25	
VORICONAZOLE	min–max	0.008–0.12	0.008	0.008–0.015	
	SD	0.11	0	0	S ≤ 0.06
	$\bar{x}$	0.04	0.01	0.01	R > 0.25
	CV	2.95	0	0.25	
ANIDULAFUNGIN	min–max	0.015–0.06	0.015–0.03	0.015–0.03	
	SD	0.02	0.01	0,01	S ≤ 0.03
	$\bar{x}$	0.04	0.02	0,03	R > 0.03
	CV	0.52	0.29	0,41	
AMPHOTERICIN B	min–max	0.25–1	0.25–0.5	0.25–1	
	SD	0.24	0.10	0.23	S ≤ 1
	$\bar{x}$	0.45	0.29	0.44	R > 1
	CV	0.54	0.36	0.52	

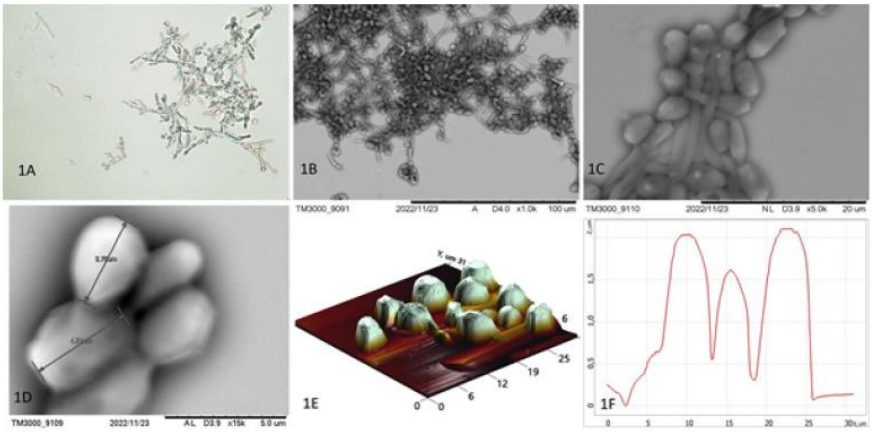
Analiza tworzenia biofilmów po 24h inkubacji wykazała, że izolaty *C. dublinensis* pozostają w formie planktonicznej. Największy potencjał do tworzenia biofilmów odnotowano w genotypie A *C.albicans* (82%) i struktury te były trwałe. W przypadku genotypu B biofilmy wykształciło 11%, a genotypu C 7%.

Podsumowanie i wnioski

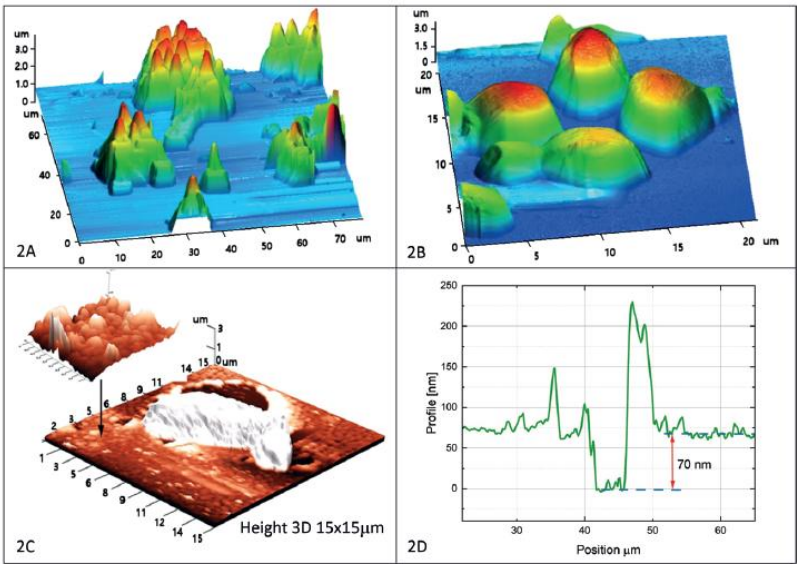
Wyniki z doświadczenia potwierdzają postanowią hipotezę 1 o tym, że izolaty drożdżaków z rodzaju *Candida*, pochodzące z jamy ustnej zdrowych osób zamieszkujących region Polski wschodniej, wykazują istotne różnice w profilu genotypowym oraz fenotypowym w porównaniu z danymi referencyjnymi dla populacji spoza tego regionu. Dodatkowo zaobserwowano, że największy potencjał do patogenezy i możliwość przetrwania niekorzystnych warunków wykazuje genotyp A *C.albicans*, najczęściej izolowany szczep z jamy ustnej osób zdrowych.

Publikacja 2 (P2) Ocena właściwości nanomechanicznych Candida albicans (Teodorowicz i wsp., 2023).

Proces tworzenia biofilmu zależy od rodzaju powierzchni i czynników środowiskowych. Tworzenie dojrzałego biofilmu obserwowano po 24-48 h na powierzchniach z polichlorku winylu i po 38-72 h na powierzchniach z polimetakrylanu metylu [Ponde i in. 2021]. W tym doświadczeniu obserwacje *C. albicans* przeprowadzono w 24-godzinnych kulturach przy użyciu mikroskopu świetlnego oraz SEM pokazały tworzenie rusztowania biofilmu oraz sieci komórek i pseudostrzępek (ryc. 1/1A-1D). Średnica komórek *C. albicans* w tej strukturze wynosiła 5,75 µm (min. 3,72 µm, maks. 7,73 µm, SD 0,66), a długość pseudostrzępek wynosiła 17,08 µm (min. 6,88 µm, maks. 40,40 µm, SD 6,85). Średnice zmierzone przy użyciu AFM (średnia wartość 4,56 mm, SD 0,84, min. 2,73, maks. 6,61) były podobne do tych uzyskanych w SEM. Wysokość komórek mierzono przy użyciu sygnału wysokości w AFM; jego średnia wartość wynosiła 2,84 mm (SD 0,29, min. 1,98 mm, maks. 3,35 mm) (ryc. 1/1E-1F; rys. 2/2A-2B). Ponadto wykryto obecność substancji zewnątrzkomórkowej otaczającej komórki *C. albicans*. W preparatach powietrzno-suchych analizowanych w AFM nie jest możliwe uwidocznienie macierzy pozakomórkowej, która wiąże komórki tworzące biofilm. Tę strukturę, która jest niezwykle ważna dla stabilności biofilmu, można zaobserwować tylko w próbkach ciekłych [Algburi i in. 2017, Le i in. 2022]. Rycina 2 przedstawia komórkę w tej strukturze o wysokości w zakresie 70-200 nm (rys. 2/2C-2D).



Rycina 1. Tworzenie się biofilmu przez *C. albicans* w 24-godzinnych hodowlach płynnych.

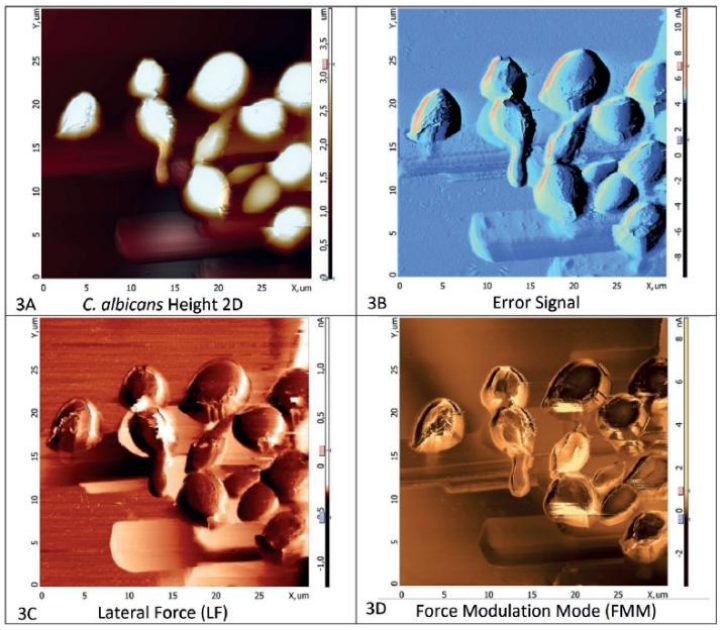


Rycina 2. Topografia komórek *C. albicans* w 24-godzinnych hodowlach z widoczną substancją pozakomórkową.

W niniejszym badaniu stwierdzono, że komórki drożdży przylegają do powierzchni szkła po 24 godzinach, a obecność mikrokolonii oraz strzępek stabilizujących struktury została wykryta. W SEM zaobserwowano zwarte, wielowarstwowe układy komórek. Zdolność do tworzenia biofilmu i wirulencja *C. albicans* są związane z przejściem od drożdży do morfotypu strzępkowego. Strzępki stabilizują biofilm i mają wpływ na jego warstwową strukturę w formach dojrzałych, podczas gdy warstwa podstawna odgrywa ważną rolę w przyleganiu biofilmu do powierzchni [Sztajer i wsp. 2014, Gulati i wsp. 2016, Lohse i wsp. 2018, Nobile i wsp. 2015, Ruszkowski i wsp. 2020].

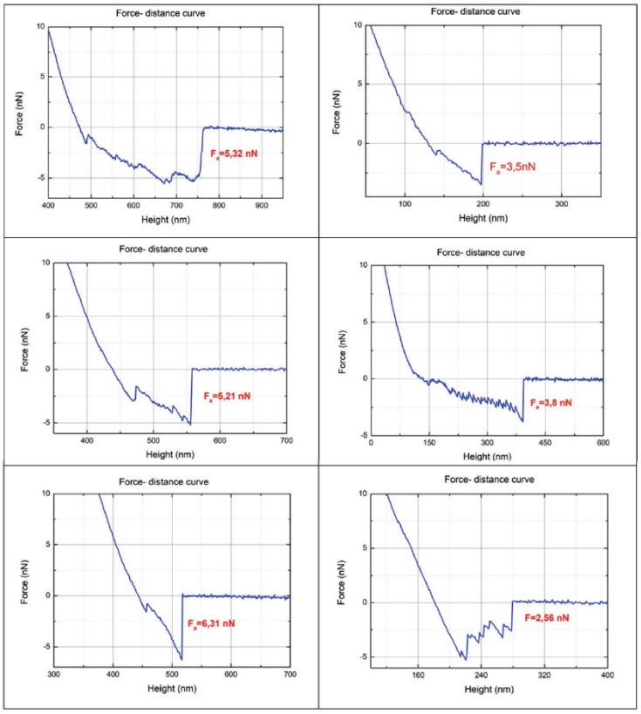
Analizy AFM patogennych mikroorganizmów, w tym grzybów, można przeprowadzać przy użyciu preparatów powietrzno-suchych i płynnych. Ta ostatnia metoda umożliwia wizualizację komórek w środowisku zbliżonym do ich naturalnych warunków wzrostu. W środowisku płynnym ściana komórkowa *C. albicans* jest gładsza i nie wykazuje zmian, które mogłyby być wywołane przez suszenie, a komórki zachowują swój naturalny turgor i elastyczność [Füzik i in. 2015]. W niniejszym badaniu zastosowano ten typ analizy AFM. Sygnał błędu AFM (ryc. 3/3B) dostarcza dodatkowych informacji jakościowych o topografii oglądanej próbki. Ułatwia on rozróżnianie małych elementów na tle dużych, stosunkowo gładkich powierzchni. W analizowanych próbkach *C. albicans* zaobserwowano obszary o różnych współczynnikach tarcia (siła boczna, sygnał LF) (ryc. 3/3C). Jasne obszary o większym tarciu (bardziej szorstkie) były widoczne w miejscach kontaktu między komórkami a szklanym podłożem. W bocznych i wierzchołkowych obszarach komórek nie zaobserwowano jasnych stref, co świadczyło o ich

mniejszej szorstkości (większej gładkości). Obszary o zwiększonej twardości (mniejszej elastyczności; sygnał FMM) znajdowano głównie w strefach komórek stykających się ze szklanym podłożem. Wierzchołkowe obszary komórek, widoczne jako ciemniejsze strefy, charakteryzowały się większą elastycznością powierzchni (ryc. 3/3D).



Rycina 3. Topografia i właściwości nanomechaniczne *C. albicans* uwidocznione za pomocą AFM (ciecz).

Siły adhezji (Fa) wyznaczone dla komórek *C. albicans* miały w każdym przypadku ten sam rząd wielkości, natomiast kształt poszczególnych krzywych różnił się od siebie. Sugeruje to stopniowe odrywanie się końcówki mikroskopu od powierzchni próbki (komórki) i wskazuje na „indywidualne" cechy powierzchni komórek biofilmu tworząc mikrokolonie (ryc. 4). Średnia wartość siły adhezji (Fa) określona dla komórek *C. albicans* obecnych w strukturze biofilmu po 24-godzinnej hodowli wynosiła 4,01 ± 1,64 nN (min. 2,56 nN, maks. 6,3 nN). Średnia wartość siły adhezji określona w niniejszym badaniu dla komórek *C. albicans* tworzących strukturę biofilmu w hodowlach 24-godzinnych wyniosła 4,01±1,64 nN.



Rycina 4. Siły adhezji (Fa) określone dla komórek *C. albicans* tworzących biofilm w hodowlach 24-godzinnych.

Podsumowanie i wnioski

Na podstawie opisanych wyników należy potwierdzić postawioną hipotezę 2. Zdolność do tworzenia biofilmu i wirulencja *C. albicans* są związane z przejściem od drożdży do morfotypu strzępkowego. Strzępki stabilizują biofilm i mają wpływ na jego warstwową strukturę w formach dojrzałych, podczas gdy warstwa podstawna odgrywa ważną rolę w przyleganiu biofilmu do powierzchni. Na wczesny etap tworzenia biofilmu wpływają oddziaływania elektrostatyczne determinujące proces adhezji komórek do substratów biotycznych lub abiotycznych, siły van der Waalsa, siły ruchu Browna i oddziaływania hydrofobowe. Istotną rolę w procesie powstawania i utrzymania struktury biofilmu odgrywa również obecność macierzy zewnątrzkomórkowej. Macierz pozakomórkowa widoczna w AFM wiąże sąsiednie komórki, a jej bezpośredni kontakt z podłożem biofilmu ma wpływ na siły adhezji.

Publikacja 3 (P3) Ocena występowania zakażeń *Borrelia burgdorferi* i *Anaplasma phagocytophilum* w kleszczach *Ixodes ricinus* pobranych od psów we wschodniej Polsce (Pańczuk i wsp., 2024).

W publikacji 3 celem badań była ocena występowania zakażeń w kleszczach *Ixodes ricinus*. W omawianym badaniu zakażenie *Borrelia burgdorferi* s.l. wykryto u 10,9% (16/147), a *A. phagocytophilum* u 12,9% (19/147) kleszczy *I. ricinus*. W jednym samcu kleszcza (0,7%)

wykazano, że był współzakażony obydwoma patogenami. Zakażenie co najmniej jednym z patogenów wykryto u 23,1% (34/147) kleszczy (Tabela 4). Obecność *B. burgdorferi* wykrywano istotnie częściej (wartość P = 0,0103) u kleszczy samców (18,2%) niż u samic (4,9%).

Częstość występowania zakażenia *A. phagocytophilum* u samców i samic była podobna, z 1,5% większą częstością u samic (13,6% w porównaniu z 12,1%). Większość kleszczy nie była napęczniała, opita w momencie pobrania (29/34). Każdy napęczniały kleszcz (5/34) został pobrany od innego bezdomnego psa przetrzymywanego w schronisku. Z tych pięciu kleszczy, jeden był zainfekowany przez *B. burgdorferi,* a cztery przez *A. phagocytophilum*. Zakażenie *Borrelia burgdorferi* było istotnie częstsze (wartość p = 0,0020) u kleszczy pobranych od psów będących w posiadaniu (18,7%) niż od psów ze schroniska (2,8%).W Polsce obecność krętków *B. burgdorferi* w kleszczach *I. ricinus* pobranych od psów jest bardzo zróżnicowana. Waha się od 0%, jak wykazano w badaniach nad psami domowymi z Zakopanego w południowej Polsce [Kocoń i wsp., 2020] do 35,7% zgłoszonych w badaniach nad psami przyjmowanymi do klinik weterynaryjnych w mieście Olsztyn w północno-wschodniej Polsce [Michalski i wsp., 2020]. Natomiast częstość występowania zakażenia *A. phagocytophilum* była podobna w obu grupach (odpowiednio 12,0% i 13,9%). Częstość występowania zakażenia *A. phagocytophilum* u kleszczy *I. ricinus* pobranych od psów lub psów i kotów w Polsce wahała się w zależności od regionu pobrania od 1,2% do 21,3%, przy czym najniższy odsetek odnotowano u kleszczy zebranych od psów przyjętych do klinik weterynaryjnych w mieście Olsztyn [Kullberg i wsp., 2020].

Podsumowanie i wnioski

Otrzymane w doświadczeniu wyniki sugerują częściowe odrzucenie postawionej hipotezy 3. Odnotowano obecność *B. burgdorferi* i *A. phagocytophilum* u kleszczy *I. ricinus* zebranych od psów w Polsce wschodniej i częstość ich występowania jest podobna, ale poziom zakażeń nie jest znacznie wyższy niż w innych regionach kraju.

Wykryta koinfekcja sugeruje możliwość równoczesnego zakażenia obydwoma patogenami poprzez pojedyncze ukąszenie kleszcza a dotychczasowe badania w Polsce rzadko zgłaszały obecność tej koinfekcji u kleszczy *I. ricinus* zarażających zwierzęta domowe.

Wskazana jest dalsza dokładna analiza występowania koinfekcji, które stają się coraz poważniejszym problemem klinicznym i bardziej potrzebnym obszarem badań, ponieważ ich

ekologia i mechanizmy patologiczne są nadal słabo zbadane w porównaniu z infekcjami pojedynczym patogenem.

Publikacja 4 (P4) Ocena występowania zakażeń *Borrelia burgdorferi* i *Anaplasma phagocytophilum* w kleszczach *Dermacentor reticulatus* pobranych od psów we wschodniej Polsce (Pańczuk i wsp., 2022).

Kleszcze pobrano od dziewięciu psów. Dziewięć kleszczy pochodziło od posiadanych psów (po jednym kleszczu od trzech psów, po dwa kleszcze od trzech innych psów), a pozostałe cztery kleszcze pochodziły od psów bezdomnych ze schroniska (po jednym kleszczu od dwóch psów, dwa kleszcze od jednego psa). Poza 13 kleszczami *D. reticulatus* zarażonymi *A. phagocytophilum*, nie znaleziono żadnych innych gatunków kleszczy u psów w momencie pobrania. Obecność *B. burgdorferi* s.l. wykryto tylko u jednego z badanych kleszczy (0,7%). Kleszcza tego pobrano od psa, który pochodził ze schroniska. W południowo-wschodniej Polsce wskaźnik zakażenia u kleszczy zebranych od zwierząt wynosił zaledwie 6,7% (tylko trzy okazy z 45 kleszczy - dwa kleszcze zebrane od kotów i jeden kleszcz od psa) były zakażone *B. burgdorferi* [Roczeń-Karczmarz i wsp., 2018].

Ocena stopnia zakażenia analizowanych kleszczy *Dermacentor reticulatus* dwoma patogenami (*Borrelia burgdorferii* s.l., *A. phagocytophilum*) wykazała, że 14 (9,2%) ze 152 badanych kleszczy było zakażonych jednym z patogenów. Nie stwierdzono współzakażeń patogenami u żadnego z kleszczy. Najwyższy odsetek zakażeń był związany z *A. phagocytophilum*. Jego obecność wykryto u 8,6% (13/152) badanych okazów. Odsetek zakażeń *A. phagocytophilum* był taki sam u kleszczy płci żeńskiej i męskiej, tj. odpowiednio 8,5% (6/71) i 8,6% (7/81). Ma to istotne znaczenie w kontekście ryzyka przenoszenia patogenów, ponieważ samce *D. reticulatus* pobierają niewielkie ilości krwi, aby zainicjować spermatogenezę, co wskazuje, że zarówno samce, jak i samice tego gatunku mogą brać udział w przenoszeniu patogenów [Bartosik i wsp., 2019; Földvári i wsp., 2016].

Podsumowanie i wnioski

W przeprowadzonym badaniu oceniono stopień zakażenia kleszczy *Dermacentor reticulatus* pobranych od dziewięciu psów, zarówno domowych, jak i bezdomnych ze schroniska. Spośród 152 analizowanych osobników 14 (9,2%) było zakażonych przynajmniej jednym z dwóch patogenów: *Anaplasma phagocytophilum* lub *Borrelia burgdorferi* sensu lato, przy czym

częstość występowania była podobna u samców i samic. Jedynie jeden kleszcz (0,7%), pochodzący od psa bezdomnego, był zakażony *B. burgdorferi* s.l. Nie stwierdzono przypadków współzakażenia tymi patogenami. Wyniki wskazują, że *D. reticulatus* może pełnić rolę wektora *A. phagocytophilum* w środowisku psów, natomiast jego znaczenie w transmisji *B. burgdorferi* s.l. wydaje się być ograniczone. Obecność zakażonych kleszczy zarówno u psów domowych, jak i bezdomnych, sugeruje konieczność monitorowania ryzyka ekspozycji na patogeny przenoszone przez kleszcze niezależnie od warunków utrzymania zwierząt.

Wnioski

Przeprowadzone badania umożliwiły charakterystykę wybranych mikroorganizmów bytujących w organizmie człowieka oraz zwierząt towarzyszących na terenie Polski wschodniej, analizując aspekty epidemiologiczne, molekularne i środowiskowe. Doświadczenia potwierdziły, że zarówno drożdżaki z rodzaju *Candida*, jak i patogeny przenoszone przez kleszcze stanowią istotne zagrożenie mikrobiologiczne w regionie Polski wschodniej.

Wykazano istotne zróżnicowanie genotypowe i fenotypowe szczepów *Candida albicans* kolonizujących jamę ustną zdrowych osób, z dominacją genotypu A, charakteryzującego się największym potencjałem patogenności, w tym zdolnością do tworzenia biofilmów i odpornością na leki przeciwgrzybicze. Ponadto zastosowanie mikroskopii sił atomowych ujawniło różnice w adhezji i właściwościach nanomechanicznych komórek *Candida*, które mają znaczenie w procesie kolonizacji i rozwoju infekcji.

Równocześnie wykazano obecność patogenów wektorowych (*Borrelia burgdorferi* s.l., *Anaplasma phagocytophilum*) u kleszczy *Ixodes ricinus* i *Dermacentor reticulatus* pobranych od psów domowych i bezdomnych. Częstość zakażeń była istotna, a u kleszczy *I. ricinus* potwierdzono nawet przypadki koinfekcji. *D. reticulatus* okazał się głównie rezerwuarem *A. phagocytophilum*, natomiast stwierdzono, że jego rola w transmisji *B. burgdorferi* s.l. była znikoma.

Uzyskane wyniki podkreślają konieczność prowadzenia stałego nadzoru nad mikroflorą ludzi i zwierząt towarzyszących, ze szczególnym uwzględnieniem czynników środowiskowych, które mogą wpływać na występowanie i patogenność analizowanych mikroorganizmów. Integracja analiz molekularnych, fenotypowych i środowiskowych pozwoliła na lepsze zrozumienie mechanizmów adaptacji, kolonizacji i zakażeń, co ma bezpośrednie znaczenie dla zdrowia publicznego, profilaktyki weterynaryjnej oraz strategii zwalczania zakażeń oportunistycznych i wektorowych.

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RESEARCH ARTICLE

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Phenotypic and genotypic characterization of *Candida* species from the oral cavity of healthy individuals in Lublin province, Poland

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ABSTRACT

Background: *Candida* spp., particularly *C. albicans*, are commonly isolated fungi in the oral cavity. However, their prevalence in healthy participants and their genotype–phenotype relation remains elusive.

Aim: This study aimed to update the information on *Candida* species colonizing the oral cavity of healthy population, identify the most common species, and characterize the intraspecific diversity to determine the genotype-phenotype relationship.

Methods: Oral swabs of healthy participants who declared an absence of oral infection were analyzed. Microbiological methods: chromogenic media, sugar assimilation tests, drug susceptibility, filamentation tests, temperature tolerance analysis, and assessment of biofilm formation ability. Genotyping methods: PCR amplification of the internal transcribed spacer (ITS) region with MspI restriction enzyme digestion and 25S rDNA region.

Results: Of the 500 individuals tested, 130 harbored *C. albicans* in 77%, *C. dubliniensis* in 12%, *Pichia kudriavzevii* (previously *C. krusei*) in 8%, and *Nakaseomyces glabrata* (previously *C. glabrata*) in 3%. The microbiological tests yielded conflicting results. Analysis of the 25S rDNA transposable intron region contributed to the identification of individual *Candida* spp. and intraspecific identification of *C. albicans* genotypes. Genotype A accounted for 70% ($n = 100$) of C. albicans isolates, whereas genotypes B, C, and D (*C. dubliniensis*) accounted for 17%, 9%, and 4% of the isolates, respectively.

Conclusion: The results indicate a complex genotype–phenotype relationship in *Candida* spp. and recommends combining microbiological and molecular methods for the efficient typing of *Candida* spp.

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Introduction

The human microbiome comprises fungal species in addition to bacteria and includes the common genus *Candida*. They have been identified in various host habitats such as the oral cavity, gastrointestinal tract, urogenital tract, and skin surface [1].

Candida spp., a genus of fungi present in the oral cavity under physiological conditions, are estimated to account for 45%–65% of the oral microbiome in healthy children and 30%–50% in healthy adults [2]. This genus is a core component of the microbiome, as it can cause oral candidiasis in the event of physiological imbalance (dysbiosis) and, consequently, may lead to systemic infections, especially in patients with immunodeficiencies.

Also the mechanism of the effect of the use of nicotine products on oral *Candida* is debatable. Substances contained in cigarette smoke may affect host immune responses, which may predispose smokers to oral infections [3]. In the literature, some studies revealed that the rate of oral Candidal carriage was higher among smokers compared with non-smokers [4]. No clear differences were noted between female and male smokers.

Candida albicans, that causes local and generalized infections, is the most frequently isolated fungal species from the oral cavity. Its ability to occupy several commensal niches is related to its virulence factors. These factors include the potential for morphological transition between yeast and hyphal forms, strong adhesion to host cells, and biofilm formation ability [5]. As organized structures, biofilms consist of closely adherent cells of one or several different species of microorganisms that are bound to the substrate and embedded in the extracellular matrix. In human body, 80% of biofilm formation is presumably associated with fungal or bacterial infections, given the durability of these structures [1,5].

Surface colonization by *Candida* when accompanied by biofilm formation is a multistage process. The first stage involves the adhesion of the cells to the substrate, providing structural stability. This process depends on many factors, including physicochemical interactions between microbial cells and the

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surface, reactions between specific fungal proteins and polysaccharides, environmental factors, chemical composition of cell membranes, specific genes, and substrate roughness [6]. The second stage is proliferation, which involves the formation of filaments, the use of which elongates the yeast cells, forming filamentous hyphae. *C. albicans* fungi can form hyphae both in planktonic cultures and at the biofilm formation stage. During this process, the fungal cells alter their morphology and become highly stable within the host cells. Hyphal growth is also regulated by transcriptional regulators and structural proteins, and the deletion of any of these elements reduces or eliminates growth [7]. The next stage is the biofilm maturation phase, in which the production of hyphae is accompanied by the secretion of extracellular polymeric substances [8,9]. The extracellular matrix, mainly composed of glycoproteins (50%), acts as a physical protective barrier to ensure the structural integrity of the biofilm. Without a matrix, fungal cells become desiccated and lose their nanomechanical properties [10]. Yeast-like cells are released from mature biofilms, leading to the formation of new structures in distant locations, leading to infection dissemination [11].

Biofilms formed by various *Candida* species differ in the complexity of their extracellular matrix structures and compositions. Mature *C. albicans* biofilms are distinguished from those produced by other *Candida* spp. because of their extensive hyphal networks. Both *C. albicans* and *C. dubliniensis*, which are difficult to distinguish using a single biochemical test, produce blastospores, hyphae, and pseudo-hyphae. Blastospores and pseudohyphae are also formed by *C. parapsilosis*, *Pichia kudriavzevii* (previously known as *C. krusei)*, and *C. tropicalis*. The fungus *Nakaseomyces glabrata* (previous *C. glabrata)* produces only blastospores [7,12,13].

C. albicans isolates are problematic as they were inconsistent in their ability to form biofilms when tested using patient samples. There are five *C. albicans* genotypes, A, B, C, D, and E, and the identification of these isolates is also troublesome. Genotype A is considered predominant, and genotype D is considered to be associated with *C. dubliniensis* [14]. Determining the genotype–phenotype relationship could predict whether a specific genotype is more likely to cause an infection. Therefore, the present study focused on determining the relationship between the *C. albicans* genotype and its phenotypic traits and biofilm-forming ability. We aimed to update our knowledge on *Candida* species colonizing the oral cavity of a healthy population. We isolated the most commonly present species and characterized the intraspecific diversity to determine the genotype–phenotype relationship.

Materials and methods

Sample collection

The study group consisted of 500 participants (288 females, 212 males) aged 18–90 years (average age, 39 years; $\sigma = 16.79$). Oral swabs were obtained from healthy volunteers with no symptoms of infection or coexisting diseases from a local community. The samples were collected between June 2021 and September 2022 in Lublin province. A questionnaire was administered to the participants to collect information on current and past oral infections, treatment methods, use of nicotine products (smoking cigarettes, e-cigarettes, and nicotine gums), morbidities, and metric data (Table 1). The study design was approved by the Bioethics Committee at Pope John Paul II State Higher School (currently John Paul II University in Biała Podlaska) (consent No. 3/2021), and all participants provided informed consent. The sample was collected using sterile swabs with a transport medium by rubbing the insides of the cheek for 15 s on each side. The sample was cultured onto Sabouraud dextrose (Argenta, Poznań, Poland) media for 24 h at 35°C. The colonies were plated on chromogenic media for further molecular analyses. Samples were banked using microbanks (Mast Group, Reinfeld, Germany), a ready to use system designed for the storage of bacterial and fungal cultures and stored at – 20°C until recovery.

Phenotypic methods

Chromogenic agar culture

After the fungi grew on Sabouraud medium, they were cultured onto chromogenic media (CHROMagar, GRASO, Poland) and incubated at 35°C for 48 h. This medium allows for the qualitative identification of yeast-like fungi of the genus *Candida*. On CHROMagar, *C. albicans* appears green, whereas *C. tropicalis* and *C. krusei* (currently *P. kudriavzevii*) appear metallic blue and pink, respectively. The color

Table 1. Participant characteristics.

	Females	Males
Number of participants	288	212
Aged 18–30 years	42% (121/288)	34% (72/212)
Aged 30–60 years	48% (138/288)	48% (102/212)
Aged above 60 years	10% (29/288)	18% (38/212)
Use of nicotine products	49% (141/288)	55% (117/212)
Country of origin	Poland	Poland
Current oral infections	0%	0%
Existing diseases	0%	0%

of *C. glabrata* (currently *N. glabrata*) ranges from violet to pink to brown, and other species are seen in shades of white to mauve-pink. Among the fungi that grew, only *C. albicans* was selected for further analyses using other phenotypic methods. Because the final results of the individual phenotypic tests were subject to unclear interpretations, a variety of analyses were performed. All results were reviewed by two independent researchers.

Sugar assimilation test

To validate the identified species in all samples, we used the AUXACOLOR 2 (Bio-Rad, Warsaw, Poland) test, a colorimetric test based on sugar assimilation. In this test, sugar utilization for yeast growth is visualized by a color change in the pH indicator from blue to yellow. Test inoculum (McFarland standard of 1.5, 100 µL), prepared from a 24-h culture, was applied to all the wells in the test apparatus and incubated at 30°C. Readings were taken after 24 and 48 h and after 72 h in case of doubt. Test results were interpreted according to the manufacturer's recommendations.

Temperature tolerance test

For the temperature tolerance test, the cultures were incubated on Sabouraud solid media at 42°C, and observations were made after 24, 48, and 72 h. The test is useful to distinguish between *C. albicans* and *C. dubliniensis*. While *C. albicans* can grow at 42°C, owing to their ability to survive unfavorable conditions, *C. dubliniensis* cannot.

Filamentation test

Fungal cells, adjusted to a McFarland standard of 2 by adding 0.5 mL human serum, were incubated at 37°C for approximately 2.5 h. Subsequently, the material was centrifuged for 3 min at 1006 × g, the supernatant was removed, and the samples were observed under a microscope (Nikon Eclipse 80i, Warsaw, Poland). The presence of filaments, that is structures characterized by non-septate cell expansion (as opposed to pseudohyphae) and lack of constriction from the mother cell, confirms the identification of *C. albicans* species (Gnat 2022).

Genotypic methods

Genomic DNA extraction

DNA isolation was performed using a Genomic Mini Kit (A&A Biotechnology, Gdańsk, Poland) according to the manufacturer's instructions. After 24 h of incubation, an inoculum with approximately 1.5 McFarland standard was prepared from Sabouraud's medium culture, transferred to Eppendorf tubes, and placed in a thermoblock heated to 70°C for 12 h to facilitate cell lysis.

Restriction fragment length polymorphism analysis

Molecular identification of the strains began with the identification of the internal transcribed spacer (ITS) region. The ITS1–5.8S-ITS2 region is characterized by relatively conserved sequences, which facilitate correct sequence alignment, and high variability, thereby ensuring the utility of non-homologous sequences as restriction fragment length polymorphism analysis markers. The following primers were selected for amplification of the targeted ribosomal DNA: ITS-1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR was carried out in a total volume of 50 µL, and the reaction mixture contained 2 µL of template DNA, 0.5 µL of each primer (25 µM), 1.25 µL of dNTPs, 5 µL of 10× PCR buffer, and 0.5 µL of Taq DNA polymerase (0.5 U). Initial denaturation was carried out for 5 min at 94°C, followed by denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and chain elongation at 72°C for 1 min in 30 cycles. The final elongation step was performed at 2°C for 7 min. Subsequently, restriction digestion was performed using the MspI restriction enzyme (10 U/µL) (Thermo Scientific, Warsaw, Poland), in a total volume of 25 µL; the digestion was achieved by incubating 21.5 µL of the PCR products with 2.5 µL of the buffer and 1 µL of the MspI enzyme. Restriction fragments were separated using 2% agarose gel electrophoresis in TBE buffer (EURx, Gdańsk, Poland) at 100 V for 1 h and stained with Gel Stain (Syngen, Wrocław, Poland) for visualization.

Genotyping using 25S rDNA

Tests for molecular differentiation of various *C. albicans* strains and between *C. albicans* and *C. dubliniensis* were carried out based on the 25S rDNA sequence. The sizes and lengths of the 25S rDNA intronic regions were determined to identify specific genotypes (A–E) of *C. albicans*. The primers CA-INT-L (5′-ATAAGGGAAGTCGGCAAAATAC CGTAA-3′) and CA-INT-R (5′- CCTTGGCTGTGG TTTCGCTAGATAGTAGAT-3′) were used for the sequence detection. The amplification reaction was carried out using 12.5 µL of Hot Start MIX (A&A Biotechnology, Gdańsk, Poland), 2.5 µL of each primer (20 pmol), and 5 µL of genomic DNA in a total volume of 25 µL.. The following conditions were used for PCR: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 s, annealing at

60°C for 15 s, extension at 72°C for 1 min, and final elongation step at 72°C for 10 min (Mashaly and Zeid, 2022). The PCR products with expected sizes (genotype A, 450 bp; genotype B, 840 bp; genotype C, 450 bp and 840 bp; genotype D, 1040 bp; genotype E, 1400 bp) were visualized using electrophoresis on a 1.5% agarose gel, using TBE buffer and Gel Stain, at 100 V for 1 h.

Data were analyzed using Statistica 13 program (Biala Podlaska, Poland). Mean, frequency, and standard deviation values, Chi-square statistic, critical value, p-value were determined. $p < 0.05$ was considered statistically significant.

Downstream analyses

Drug susceptibility assessment

Antifungal susceptibility testing was performed on isolates confirmed as *C. albicans* (genotypes A, B, or C) to the commonly used antifungals anidulafungin, amphotericin B, micafungin, posaconazole, voriconazole, itraconazole, and fluconazole. To examine the drug susceptibility profile, minimum inhibitory concentrations (MICs) of the selected agents were determined using a microbroth dilution test in a dried 96-well microplate format. Each well contained antifungal agents at appropriate dilutions and a calorimetric indicator (Sensititre YeastOne; Thermo Scientific, Warsaw, Poland). The plate was incubated at 35°C for 24 or 48 h in case of doubt. The lowest concentration of the antifungal agent that inhibited fungal growth (as evidenced by the lack of color change) was recorded. Strains were considered sensitive or resistant according to EUCAST recommendations [15].

Biofilm formation analysis

Cultures incubated at 35°C for 48 h were used for biofilm observation. A microscope slide coated with gelatin was placed in a sterile container and supplemented with 2 mL of the fungal inoculum (with a McFarland standard of 4) and 2 mL of Sabouraud dextrose liquid medium (Oxoid, Pol-Aura, Warsaw, Poland). After incubation, non-adherent and planktonic cells were removed by washing with PBS (phosphate-buffered saline) (1.5 mL). Biofilms were visualized and analyzed using a Nikon optical microscope (Eclipse 80i model) equipped with a Nikon DS-Fi1c camera at 40× magnification. Most biofilms formed under *in vitro* laboratory conditions have only a few layers and cells that adhere well to the substrate and are resistant to repeated washing. The degree of biofilm formation by each isolate was determined as follows. After rinsing, a biofilm occupying more than 80% of the field of view with complex structures was defined as a strong biofilm; if it occupied 50% of the area with few cells, it was described as an intermediate biofilm, and a biofilm with an occupancy below this value was identified as a weak biofilm.

Results

Candida spp. were detected in the oral swab samples of 130 of the 500 participants, indicating that 26% of individuals harbored the fungi (Table 2). Of the 130 *Candida*-positive samples, 77% (100/130) carried *C. albicans*, 12% (16/130) contained *C. dubliniensis*, 8% (10/130) contained *P. kudriavzevii*, and 3% (4/130) contained *N. glabrata*, as revealed by phenotypic analyses such as the CHROMagar identification. In this study, no more than one species was isolated from a single individual.

Statistical analyses were performed used Statistica 13 to determine significant differences between male and female participants who tested positive for *Candida* spp. and used nicotine. The null hypothesis assumed no significant difference in the distribution of the number of individuals harboring *Candida* spp. and nicotine use between men and women. The values of the chi-square statistic ($\chi^2 = 3.519$) were compared with the critical value (3.841) at $df = 1$; at the pre-determined significance level ($\alpha = 0.05$), there was no difference ($p = 0.061$) in the prevalence of *Candida* spp. and nicotine use between men and women.

Further phenotypic identification tests were performed on the 100 isolates identified as *C. albicans* using chromogenic media. Results are summarized in Table 3.

Table 2. Characteristics of the 130 individuals who harbored *Candida* spp.

	Females	Males	Statistical calculations
Samples harboring Candida spp.	63% (82/130)	37% (48/130)	
Aged 18–30 years	38% (31/82)	32% (15/48)	
Aged 30–60 years	54% (44/82)	49% (24/48)	
Aged above 60 years	8% (7/82)	19% (9/48)	
Use of nicotine products	17% (14/82)	32% (15/48)	$\chi^2 = 3.519$ $df = 1$ $\alpha = 0.05$ p-value = 0.061

Table 3. Phenotypic test results using the AUXACOLOR sugar assimilation, temperature tolerance, filamentation, and sensititre YeastOne tests for the 100 isolates initially classified as *C. albicans*.

	C. albicans	C. dubliniensis
AUXACOLOR sugar assimilation test	97% (97/100)	3% (3/100)
Temperature tolerance test	93% (93/100)	7% (7/100)*
Filamentation test	97% (97/100)	3% (3/100)**
Sensititre YeastOne test	100% (100/100)	0% (0/100)

*Results of three AUXACOLOR tests considered; **For one isolate, the results were validated using temperature tolerance and AUXACOLOR tests as well.

PCR products, of sizes ranging from approximately 510 to 870 bp, specific for *Candida* spp. were identified by analyzing the ITS region. After cleaving the ITS region with the MspI restriction enzyme, two polymorphic bands were obtained for *C. albicans*, *C. dubliniensis*, *N. glabrata*, and *C. tropicalis*, and one band was obtained for *P. kudriavzevii*. The similarity in the restriction patterns of *C. albicans* and *C. dubliniensis* prevented proper differentiation of the two strains (Figure 1).

All 100 strains that were isolated and tentatively identified as *C. albicans* and/or *C. dubliniensis* were subjected to PCR analysis based on the 25S rDNA sequence for genotyping; a single product was obtained for genotypes A (450 bp) and B (840 bp), and a double product was obtained for genotype C (450 bp and 840 bp). The following genotypes were identified (Figure 2): genotype A (70%), B (17%), C (9%), and D (*C. dubliniensis*, 4%). All analyses for genotype D yielded a band identical to that of *C. dubliniensis* (1080 bp), which contradicted the results obtained using sugar assimilation and filamentation tests. Genotype E was not detected in the present study. Further, the results revealed no relationship between the occurrence of a given genotype and nicotine use.

The 96 isolates confirmed as *C. albicans* (genotype A, B, or C) were tested for antifungal susceptibility.

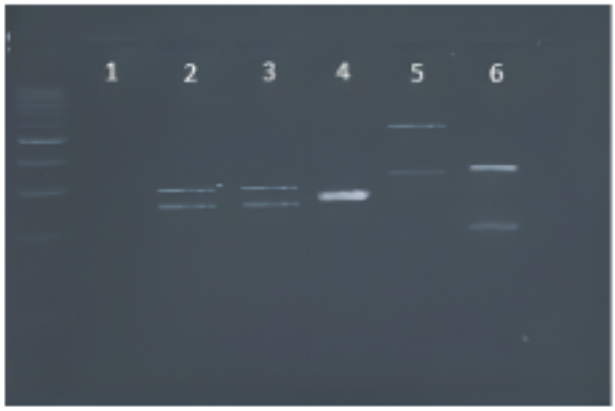

Figure 1.Band patterns of the PCR products of the ITS1–5.8S-IT2 region after MspI restriction digestion for *Candida* spp. visualized using gel electrophoresis; lane 1, negative control; lane 2, *C. albicans*; lane 3, *C. dubliniensis*; lane 4, *P. kudriavzevii*; lane 5, *N. glabrata*; and lane 6, *C. tropicalis*.

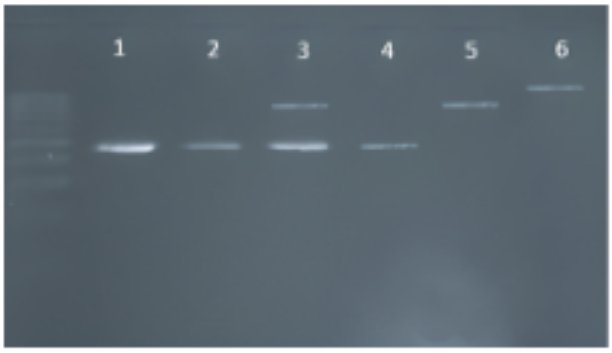

Figure 2.Genotyping DNA band pattern profiles. Lanes 1, 2, and 4, *C. albicans* genotype A; lane 3, *C. albicans* genotype C; lane 5, *C. albicans* genotype B; lane 6, *C. dubliniensis*/*C. albicans* genotype D.

The results are summarized in Table 4, along with the MICs of the agents used for testing. All samples identified as genotypes B or C were susceptible to all the antifungal drugs. Genotype A samples were highly resistant, with 57% (40 of 70) of the isolates showing resistance to anidulafungin. In addition, resistance to posaconazole, itraconazole, and voriconazole was observed in 5 (7.1%), 15 (21.4%), and 4 (5.71%) isolates, respectively. However, all the genotype A samples were susceptible to fluconazole, micafungin, and amphotericin B.

Among the isolates classified as *C. albicans*, 65% (62 of 96) formed biofilms (Figure 3) and 35% (34 of 96) were present in the planktonic form. The genotype distribution of the isolates in the biofilm-forming group was as follows: 82% (51 of 62) of genotype A, 11% (7 of 62) of genotype B, and 7% (4 of 62) of genotype C.

Six strains, with five representing genotype A (83%) and one representing genotype C (17%), formed strong biofilms. Genotype D isolates did not form biofilms.

Discussion

The development and recurrence of fungal infections of the oral cavity are closely associated with the presence of *Candida* spp. in the microbiome. The present study revealed *Candida* yeast colonization in the oral cavity of 26% of the examined participants, which is approximately 13% lower than the prevalence reported by Gerós-Mesquita et al. [16] in a similar study with healthy participants. In the study by Szymańska et al. [17], the incidence of yeast in the oral cavity of healthy participants was 30%.

C. albicans is the most common species in this environment owing to its features such as high pathogenicity, virulence, and adaptability [2,18,19]. Our findings re-affirmed this result, with *C. albicans* still being the most frequently (77%) isolated species of

Table 4. MIC values (mg/l) of the antifungal agents against the tested *Candida albicans* strains (genotype A, B, or C).

	Candida albicans species			
	Genotype A	*Genotype B*	*Genotype C*	
Number of isolates (n = 96)	70 (73%)	17 (18%)	9 (9%)	MIC breakpoint
Antifungal agents used for testing		MIC values (mg/L)		(mg/L)
FLUCONAZOLE min–max	0.12–1	0.12–0.5	0.12–1	S ≤ 2
SD	0.20	0.10	0.25	R > 4
x̄	0.27	0.29	0.37	
CV	0.76	0.34	0.67	
POSACONAZOLE min–max	0.15–0.8	0.015–0.3	0.008–0.3	S ≤ 0.06
SD	0.02	0.01	0	R > 0.06
x̄	0.02	0.02	0.02	
CV	0.94	0.65	0.28	
ITRACONAZOLE min–max	0.015–0.5	0.015–0.06	0.015–0.06	S ≤ 0.06
SD	0.02	0.01	0.02	R > 0.06
CV	0.04	0.03	0.04	
	0.44	0.38	0.42	
MICAFUNGIN min–max	0.008–0.03	0.008–0.015	0.008–0.015	S ≤ 0.016
SD	0.01	0	0	R > 0.016
x̄	0.01	0.01	0.01	
CV	0.51	0.29	0.25	
VORICONAZOLE min–max	0.008–0.12	0.008	0.008–0.015	S ≤ 0.06
SD	0.11	0	0	R > 0.25
x̄	0.04	0.01	0.01	
CV	2.95	0	0.25	
ANIDULAFUNGIN min–max	0.015–0.06	0.015–0.03	0.015–0.03	S ≤ 0.03
SD	0.02	0.01	0,01	R > 0.03
x̄	0.04	0.02	0,03	
CV	0.52	0.29	0,41	
AMPHOTERICIN B min–max	0.25–1	0.25–0.5	0.25–1	S ≤ 1
SD	0.24	0.10	0.23	R > 1
x̄	0.45	0.29	0.44	
CV	0.54	0.36	0.52	

SD: standard deviation (mg/L); x̄: arithmetic average (mg/L); CV: coefficient of variation (%); S (susceptibility breakpoint); R (resistance breakpoint).

Figure 3.Images of biofilms formed *in vitro* by *C. albicans* genotype a isolates. (A) Strong biofilm, (B) intermediate biofilm, (C) weak biofilm.

this genus. The high prevalence in healthy participants who declared no oral infections indicates that its presence in patients with compromised immunity or chronic inflammation in the oral cavity may lead to the development of infection and colonization of other niches in the body.

Our research hypothesis assumed a significant influence of external factors on colonization in the oral cavity but in research we did not observe a correlation between increased yeast colonization of the oral cavity and the use of nicotine products. Among people without *C. albicans* colonization, the majority of participants smoked across all the age groups. In addition typically, the functions of the innate immune system are impaired in elderly individuals aged >60 years. Surprisingly, in our study, *C. albicans* isolates were mostly identified in samples collected from participants aged 30–60 years, with a slight predominance in females.

Proper identification of the pathogen is important for the implementation of appropriate treatment and antibiotic therapy. Different methods have been described for *Candida* spp. identification and genotyping [14,20,21]. The choice of typing method depends on the nature and aims of the molecular study, and the efficiency of the technique is determined by its discrimination power, repeatability, accuracy, ease of performance, and interpretability of results. In the present study, phenotypic methods, even when using multiple techniques, may yield

ambiguous results and cannot be performed selectively. We demonstrated that a single method is insufficient for the identification of *Candida* fungi, especially to discriminate between *C. albicans* and *C. dubliniensis*, owing to their common phenotypic traits. However, microbiological methods have limitations. Often, the interpretation of results is visual (based on color readings), and the results may be subjective. These disadvantages of phenotype-based techniques for *Candida* spp. identification were confirmed in the present study. Results must be reproducible and interpreted identically, regardless of the handling team, and hence, molecular methods, such as PCR-based methods, are currently used to complement microbiological methods and provide accurate results. In our study, the species identified as *C. albicans* using microbiological techniques was reassigned as *C. dubliniensis* on genomic analysis.

Progress in molecular biology has facilitated its use in pathogen typing. The distinction of *Candida* spp. in the oral cavity based on the ITS region and digestion of PCR products with the MspI restriction enzyme revealed patterns for the easy identification of *P. kudriavzevii*, *N. glabrata*, and *C. tropicalis*. The electrophoretic results for *C. albicans* and *C. dubliniensis* were almost identical. This is not surprising considering the limitations associated with phenotypic identification. These results suggest that the strains are strongly interrelated, as confirmed by other researchers such as Zahir and Himratul-Aznita [22]. In the present study, ITS method proved efficient for the analysis of strains in the microbiome of healthy participants, as it revealed the interspecific differentiation of *P. kudriavzevii*, *C. tropicalis*, *N. glabrata*, and *C. albicans/C. dubliniensis*. However, the drawback with respect to the identification of *C. albicans* and *C. dubliniensis* could have been overcome by the selection of a different restriction enzyme, for example BlnI, as shown by Shokohi et al. [23].

Genotypic methods also facilitate the identification of the same or closely related strains in independent isolates, assessment of their microevolution and variability, and recognition of unrelated strains. A relatively easy, quick, and reliable genotyping method was used to analyze changes in the length of the 25S rDNA transposable intron, which allowed interspecific and intraspecific differentiation of *C. albicans* based on band patterns. The high variability and intraspecific diversity of *C. albicans* was evidenced by the presence of genotypes A, B, and C. The findings from the present study showed that the oral cavity of most healthy participants were colonized by genotype A (70%), followed by genotypes B (17%), C (9%), and occasionally genotype D identified as *C. dubliniensis* (4%). Further, no two genotypes were simultaneously identified in any participant. Similar results have been reported by Mashaly and Zeid [24] and She et al. [25]. Compared with the results of our research, other results were obtained by Tantivitayakul et al. [14]. In this report found that genotype B was the most common genotype in a pool of participants from Thailand. Researchers suggest the influence of geographical location on the distribution of genotypes.

C. albicans isolates classified as genotypes B and C were susceptible to all the antifungal agents used for testing, including all the echinocandins, whereas genotype A was resistant to anidulafungin, posaconazole, itraconazole, and voriconazole. This may be related to the prevalence of genotype A in the healthy population and its higher adaptability than those of the less common genotypes B and C, leading to acquired resistance. Resistance of the common *C. albicans* genotype A to azole and echinocandins antifungals may contribute to treatment failure [26]. This is due to the fact that they constitute the basic groups of drugs used in potential use in therapy of Invasive fungal disease. Further, our findings differ from those of Kumar et al. [27], in which *C. albicans* genotype B was resistant to most echinocandins. A low percentage of *C. albicans* is reportedly resistant to antifungal drugs by Kessler et al. [28]. The highest resistance rates were observed against miconazole and econazole, regardless of the presence of systemic disease. Comparing this with the results of our study, the differences may be caused by the geographical region of the carriers (Brazil/Poland) or the genotypes of the individual strains. This may also be attributed to the diversity of microorganisms colonizing the oral cavity.

rDNA analyses, in combination with the assessment of other pathogenic traits such as filamentation, colony morphology, and drug sensitivity, revealed that high intraspecific diversity was related to the ability to form biofilms. As confirmed in the present study, each genotype was characterized by different biofilm-forming abilities. Biofilms were formed by 43% (highest proportion) of genotype A, 11% of genotype B, and 7% of genotype C isolates, with strong substrate adhesion exhibited by biofilms formed by the first and third genotypes. Additionally, genotype A exhibited the highest filamentation ability among all genotypes, which could be associated with the extreme ease of creating complex structures by these microorganisms. Nevertheless, isolates that could not form biofilms were also observed, even those belonging to genotype A, which might be related to the individual characteristics acquired from the host.

Conclusions

The present study showed that microbiological methods have limitations and that they should be

combined with molecular methods for efficient typing of *Candida* spp. ABC genotyping revealed that the predominating genotype is A among the *C. albicans* strains isolated in our study, which is probably associated with its other traits: extensive colonization of the oral cavity of healthy people, ease of biofilm formation, strong adhesion to the substrate, high resistance to antifungal drugs, intense filamentation, and high adaptability. The importance of the genotype-phenotype relationship in *Candida albicans* genotype A is well known complemented by studies that have provided deeper insight into the pathogenicity and development of fungal infections with the strains most commonly found in the oral cavity, even in healthy individuals.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Białe Podlaska, 22.07.2025

Data i miejsce

Patrycja Andrzejuk

Imię i nazwisko

Centrum Badań nad Innowacjami Akademii Bialskiej im. Jana Pawła II

Afiliacja

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Teodorowicz P, Tokarska – Rodak M, Michaluk E, Zarębska M, Plewik D, Grudniewski T, Sacharczuk M. Assessment of nanomechanical properties of Candida albicans as an element of the oral mycobiota in healthy subjects : a preliminary study. 2023. Animal Science Papers and Reports 41.2: 165-178. DOI: 10.2478/aspr-2023-0006

mój udział polegał na:

wykonywaniu doświadczeń, zebraniu i analizie danych, pomocy w przygotowaniu artykułu (30% wkładu)

Patrycja Andrzejuk

Podpis

22.01.2025 Biała Podlaska

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mój udział polegał na:

zaplanowaniu doświadczeń, analizie i interpretacji danych, krytycznej rewizji i opracowaniu artykułu (30% wkładu)

Małgorzata Tokarska - Rodak

Podpis

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OŚWIADCZENIE WSPÓŁAUTORA

Oświadczam, że w pracy:

Teodorowicz P, Tokarska – Rodak M, Michaluk E, Zarębska M, Plewik D, Grudniewski T, Sacharczuk M. Assessment of nanomechanical properties of Candida albicans as an element of the oral mycobiota in healthy subjects : a preliminary study. 2023. Animal Science Papers and Reports 41.2: 165-178. DOI: 10.2478/aspr-2023-0006

mój udział polegał na:

wykonywaniu doświadczeń, zebraniu i analizie danych, pomocy w przygotowaniu artykułu (20% wkładu)

Estera Michaluk

Podpis

Białe Podlaskie, 10.07.2025 r.

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Afiliacja

OŚWIADCZENIE WSPÓŁAUTORA

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Assessment of nanomechanical properties of *Candida albicans* as an element of the oral mycobiota in healthy subjects – a preliminary study

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In a healthy physiological state, the mucous membrane of the oral cavity creates a suitable environment for the colonization of *Candida* spp. yeasts. The aim of the study was to analyze the nanomechanical properties of *C. albicans* cells derived from the oral cavity of healthy people in a biofilm produced in laboratory conditions. *Candida* spp. were sampled from the oral cavity of healthy individuals. The process of biofilm formation was analyzed using classic microscopic observation enriched with SEM (scanning electron microscope) and the nanomechanical properties of the cells were assessed with the use of the atomic force microscopy technique (AFM). From all isolated strains in the samples collected of the oral cavity healthy people was detected 79% *C. albicans*. Other isolated species belonged to the group „non-albicans". The observations of *C. albicans* carried out in 24-h cultures revealed a tendency of the cells to form a biofilm structure

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with multilayer cell systems. The diameter of *C. albicans* cells in this structure was 5.75 µm, and the length of the pseudohyphae was 17.08 µm. The presence of an extracellular substance surrounding the *C. albicans* cells was detected. The mean value of the adhesion force determined for *C. albicans* cells was 4.01 nN. Areas with increased hardness (Force Modulation Mode signal; FMM signal) were found mainly in the zones of cells in contact with the glass substrate. The analysis of *Candida* cells in liquid samples gives satisfactory results, as it prevents unfavorable changes in the cell surface and thus provides more reliable results. The quality of the biofilm is probably related to the nanomechanical properties of *C. albicans* cells and may consequently contribute to the stability of the biofilm structures and their susceptibility or resistance to antifungal drugs.

The presence of *Candida* spp. especially in companion animals (dogs, cats) poses a risk of their transmission to the human organism. For this reason, it is advisable to undertake additional research to analyze the ability of zoonotic-origin *Candida* spp. to form biofilms with comparison of the biofilm-formation capacity of species isolated from humans.

KEY WORDS: yeast / mycobiota / AFM / healthy subjects

The composition of the microbiota of the skin, oral cavity, and gastrointestinal and urogenital tracts varies, as these regions are colonized by a varying number and species composition of bacteria, archaea, bacteriophages, and fungi. The oral cavity is a unique environment for the growth and persistence of many of these microorganisms due to the specific possibility of contact with the external environment. It is characterized by a highly diversified microbial composition, as the bacterial and fungal microbiocenoses of the dental plaque, tongue surface, and saliva may differ substantially between each other. In healthy people, elements of the microbiota and mycobiota exhibit a delicate balance [Nobile *et al.* 2015, Gulati *et al.* 2016, Malinowska *et al.* 2017, Vesty *et al.* 2017, Ruszkowski *et al.* 2020]. Fungi present in the oral cavity of healthy individuals have been most often identified to represent the genera *Candida, Alternaria, Cladosporium, Aspergilllus, Cryptococcus, Fusarium, Auerobasidium, Aureobasidium,* and *Malassezia* [Nobile *et al.* 2015, Ruszkowski *et al.* 2020, Ponde *et al.* 2021, Pristov *et al.* 2019]. Species from the genera *Saccharomyces*, *Penicillium*, *Scopularis*, and *Geotrichum* are important elements of oral mycobiota as well [Malinowska *et al.* 2017].

Candida albicans, i.e. the most frequent representative of the oral mycobiota, can asymptomatically colonize the oral cavity in healthy people. Simultaneously, this opportunistic pathogen can cause infections of varying severity in certain conditions [Sztajer *et al.* 2014, Lohse *et al.* 2018, Pristov *et al.* 2019, Ponde *et al.* 2021]. In addition to *C. albicans*, the other opportunistic species identified in healthy individuals include *C. glabrata, C. tropicalis, C. krusei*, and *C. parapsilosis* [Pristov *et al.* 2019]. Immune disorders associated with infections and cancer diseases, stress, treatment with immunosuppressive drugs and broad-spectrum antibiotics, post-surgical treatment conditions, changes in diet, nutritional deficiencies [Pieczyńska *et al.* 2020, Yeung *et al.* 2021, 2022], and changes in the oral environment (temporary changes in pH, changes in oxygen levels) can cause an imbalance within the microbiome and promote *Candida* proliferation. This, consequently, may lead to development of various fungal infections from mucous membrane infections (candidiasis) to candidemia [Nowak *et al.* 2009, Lohse *et al.* 2018, Pristov *et al.* 2019, Ponde *et al.* 2021].

The ability of microorganisms to form biofilms is their adaptation to colonize specific environmental niches and, from the medical point of view, a virulence factor leading to the development of infection. Biofilms can form on various biotic (oral cavity, respiratory tract, genitourinary tract) and abiotic (dentures, dental implants, catheters) surfaces [Lal *et al.* 2008, Gulati *et al.* 2016, Pristov *et al.* 2019, Jafri *et al.* 2020, Ponde *et al.* 2021]. They are formed by many *Candida* species (C. *glabrata, C. krusei, C. parapsilosis*, and *C. tropicalis*); however, *C. albicans* is the most common biofilm producer in clinical settings [Pristov *et al.* 2019]. These fungi can form mixed biofilms with bacterial microorganisms involved in the development of inflammatory changes in the oral cavity and periodontium, e.g. *Streptococcus* group oralis - *Streptococcus mutans, Streptococcus gordonii, Staphylococcus aureus, Actinomyces viscosus, and Fusobacterium spp.* [Gulati *et al.* 2016]. *Together with S. mutans, C. albicans* can form dental plaque. A synergistic partnership was observed between *S. oralis*, *S. sanguinis*, and *C. albicans*, where the fungus promoted biofilm formation by the streptococci on abiotic surfaces and on the mucosa [Jafri *et al.* 2020]. *Candida* biofilms are a serious clinical problem, as the fungi are resistant to antifungal drugs and environmental factors [Sztajer *et al.* 2014]. They are also a challenge to the host defense mechanism and can be a source of infection spreading through the bloodstream [Sztajer *et al.* 2014, Gulati *et al.* 2016, Lohse *et al.* 2018].

The aim of the study was to analyze the nanomechanical properties of *C. albicans* cells derived from the oral cavity of healthy people in a biofilm produced in laboratory conditions. The process of biofilm formation was analyzed using SEM (scanning electron microscope) observations and the nanomechanical properties of the cells were assessed with the use of the atomic force microscopy technique (AFM).

Material and methods

Origin of samples, identification of *Candida* spp., and determination of the MIC value

Candida spp. were sampled from the oral cavity of healthy people as part of the research projects: "Occurrence of *Candida* spp. fungi in the oral cavity microflora in physiological balance conditions" and "Analysis of the genetic relationship of *Candida* spp. strains found in the oral microbiota of healthy individuals under physiological balance conditions" (consent of the bioethics committee; Pope John Paul II State School of Higher Education in Biała Podlaska No. 9/ 2018 and No.3/2021/current name of the university: John Paul II University of Applied Sciences).

In laboratory conditions, swabs from the buccal surface were taken (once for each person) from adults declaring the absence of oral cavity infections. The cultures were carried out on Sabouraud agar medium with gentamicin and chloramphenicol (Oxoid) at 35°C for 48 h. The strains were preserved until analysis in Viabank™ for long-term storage of microorganisms at low temperatures (Medical Wire & Equipment Co). The strains were then revived in Sabouraud medium (incubation for 24 h, 35°C).

Candida spp. species were identified using the Auxacolor™ 2 kit (Bio-Rad). A 24-hour culture on solid Sabouraud medium with gentamicin and chloramphenicol (Oxoid) was used for the assays. A suspension with McFarland turbidity of 1.5 was transferred with 100 µl of inoculum to each microplate well, sealed with adhesive tape, and incubated at a temperature of 30°C. The readings were carried out every 24 h in accordance with the manufacturer's instructions, and the final interpretation was performed after 72 h.

The MIC values (minimum inhibitory concentration) for fluconazole (FLU), itraconazole (ITC), posaconazole (POS), voriconazole (VO), anidulafungin (AND), micafungin (MYC), and amphotericin B (AMB) against the analyzed *Candida* spp. strains were determined using MTSTM gradient strips and RPMI agar medium (Liofilchem®). In accordance with the manufacturer's instructions, the MIC values were read in 24-h cultures (35°C). The values were referred to the EUCAST guidelines [EUCAST 2023].

C. albicans preparations for light, SEM, and AFM microscopy

50 ml of *C. albicans* culture (35°C; 48h) in Sabouraud liquid medium with dextrose (Oxoid) was centrifuged (8 min 2000 x g). The supernatant was decanted and 30 ml of PBS was added, mixed by inversion, and centrifuged again (8 min 2000 x g). Next, 30 ml of PBS was added to the precipitate, and the suspension was adjusted to 4 McFarland turbidity. Four 1-cm^2 sterile slides were placed on the bottom of a 100-ml sterile container, and 16 ml of the *C. albicans* suspension in PBS was added to obtain a 1-cm layer of the liquid above the slide [Füzik *et al.* 2015]. The samples were incubated to induce adhesion (37°C; 90 min; 75 RPMI rocking shaker), and the slides were rinsed gently by immersion in PBS (10 ml) [Lal *et al.* 2008]. The slides were then placed in liquid Sabouraud medium (16 ml) and incubated at 37°C with 75 RPMI. After 24-h incubation, the slides were removed with sterile tweezers, rinsed three times with PBS (1 ml), and viewed using a light microscope (Nikon, Eclipse Ni-U model; Nikon DS-Fi1c camera; NIS Elements D software; x40 magnification), SEM, and AFM. SEM measurements were performed on a scanning electron microscope Hitachi model TM 3000 equipped with an energy - dispersive X-ray spectroscopy (Oxford Instruments). We used backscattered electron imaging with accelerating voltage of 15kV. *Candida* samples were prepared on 25 x 25 mm quartz coated glass substrates (Ossila, UK) and dried. Then, a thin (fiew nanometers) layer of 99.999% pure copper was sputtered onto them (Testbourne, UK). The Cu layer was deposited with a DC magnetron sputtering system (nano PVD, Moorfield) in an atmosphere of pure argon (99.999%) at a pressure $1{,}8 \cdot 10^{-2}$ mbar.

AFM technique was used to determine in nanoscale local mechanical properties of the samples (based on the force-distance curve) and their 3D topography. Images were collected using a NTEGRA Spectra C microscope (NT-MDT) operating in contact mode (Height, Lateral Force, Error Signal, Force Modulation Mode). In order to provide the samples with physiological conditions, all measurements were

performed in liquid, at room temperature (21°C). All images were acquired using very soft, triangular Si_3N_4 cantilevers designed for imaging of biological samples (MSCT Bruker) with a nominal spring constant of k~0.01 N/m and resonance frequency f_o~7 kHz. Force measurements were conducted by positioning the tip through AFM images over individual *Candida* cells. The data analysis of the 2D surface topographies as well as the 3D representation of the AFM images were performed with the aid of NOVA 1.1.0.1824 software.

The results were analyzed statistically (Statistica v.10 software). The maximum and minimum values, arithmetic average and standard deviations (SD) were calculated.

Results and discussion

The composition of the oral mycobiome varies depending on the analyzed niche. As reported by Vesty, the dental plaque mycobiome was dominated by *Candida* species (>99% of sequences), with the highest proportion of *C. albicans*. In turn, *C. dubliniensis* and *C. tropicalis* were detected less frequently. In saliva, the analysis of sequences revealed the presence of >50% of *Candida*, 6-19% of *Penicillium,* <10% of *Saccharomyces,* and <1% of *Malassezia*; 14-20% of the sequences were not identified to the genus level [Vesty *et al.* 2017]. Other studies of the oral mucosa identified *C. albicans* in 40% of healthy subjects, whereas non-*C. albicans* species were represented by *C. parapsilosis* (15%), *C. tropicalis* (15%), *C. khmerensis*, and *C. metapsilosis* (5%) [Ghannoum *et al.* 2010]. The presence of *C. albicans* was detected in supragingival plaque in 12% of children [Ruszkowski *et al.* 2020] and on the buccal and palate surfaces in 56.5% of healthy adults [Kimsa *et al.* 2020]. The presence of 26 (79%) *C. albicans* strains, 4 (12%) *C. glabrata* strains, 2 (6%) *C. krusei* strains, and 1 (3%) *C. dublinensis* strain was detected in the samples collected from the oral cavity of the healthy subjects. The MIC values of the tested antifungal agents are presented in Table 1. The results were interpreted in accordance with the EUCAST recommendations. All *C. albicans* strains were susceptible to fluconazole (FLU), voriconazole (VO), anidulafungin (AND), micafungin (MYC), and amphotericin B (AMB). Based on the susceptibility to echinocandins, it can be concluded that all strains were also sensitive to caspofungin. Resistance to itraconazole (ITC) and posaconazole (POS) was exhibited by 3 strains (11.5%) and 1 (3.8%) strain, respectively.

All the *C. glabrata* strains were classified as susceptible to the exposure to fluconazole (FLU). The species is characterized by naturally reduced susceptibility to fluconazole, and the present results indicate that the strains do not exhibit acquired fluconazole resistance mechanisms. All the *C. glabrata* strains were sensitive to echinocandins and amphotericin B (AMB). The EUCAST and CLSI recommendations do not provide interpretation of the susceptibility of *C. glabrata* to the other azoles.

C. krusei fungi are naturally resistant to fluconazole (FLU); therefore; regardless of the MIC values, all strains were considered resistant. In the case of this species, the susceptibility to only anidulafungin (AND) and amphotericin B (AMB) can

Table 1. MIC values (mg/L) of the tested *Candida* spp. strains

Species		*C. albicans*	*C. glabrata*	*C. krusei*	*C. dublinensis*
Number of strains tested (N=33)		26 (79%)	4 (12%)	2 (6%)	1 (3%)
Antifungals		MIC			
FLU	min-max	0.19-1	0.75-8	8-96	0.38
	median	0.3	7	52	0.38
ITC	min-max	0.016-0.094	0.094-1.5	0.38-0.5	0.047
	median	0.063	0.75	0.44	0.047
POS	min-max	0.016-0.094	0.064-0.75	0.19-0.25	0.023
	median	0.048	0.625	0.22	0.023
VO	min-max	0.002-0.016	0.032-0.125	0.125-0.25	0.008
	median	0.005	0.094	0.188	0.008
AND	min-max	0.006-0.032	0.047-0.064	0.032-0.064	0.016
	median	0.018	0.064	0.048	0.016
MYC	min-max	0.004-0.008	0.006-0.006	0.064-0.064	0.008
	median	0.006	0.006	0.064	0.008
AMB	min-max	0.19-0.38	0.25-0.5	0.5-1	0.047
	median	0.25	0.38	0.75	0.047

FLU – fluconazole; ITC – itraconazole; POS – posaconazole; VO – voriconazole; AND – anidulafungin; MYC – micafungin; AMB – amphotericin B.

be interpreted using EUCAST. Both strains were sensitive to these antibiotics; additionally, their susceptibility to anidulafungin can evidence their susceptibility to caspofungin. Furthermore, the CLSI guidelines facilitated interpretation of the values for micafungin (MYC) and voriconazole (VO): the analyzed *C. krusei* strains were sensitive to these antibiotics. No interpretation was possible in the case of the other antibiotics.

The *C. dublinensis* strain was sensitive to azoles and amphotericin B (AMB). No interpretation was possible in the case of the other drugs.

Azoles, polyenes, echinocandins, and nucleoside analogs are the main classes of antifungal drugs used in clinical practice. However, biofilms formed by *C. albicans* are often resistant to their action due to the properties of the extracellular matrix, the existence of metabolically inactive "persister" forms, and the upregulation of efflux pumps. These factors hamper effective elimination of *Candida* biofilms [Nobile *et al.* 2015, Gulati *et al.* 2016, Perlin *et al.* 2017, Lohse *et al.* 2018, Gao *et al.* 2018]. Azoles are the largest group of antifungal drugs widely used in clinical practice. However, the resistance of *Candida* to drugs from this group has been reported to increase, hence the increasing importance of echinocandins in the treatment of *Candida* infections [Pristov *et al.* 2019]. Mature *C. albicans* biofilms are highly tolerant to amphotericin B, fluconazole, and caspofungin, which are effective in elimination of planktonic cells [Ponde *et al.* 2021]. One of the components of the extracellular matrix of *C. albicans* biofilms, i.e. the polysaccharide β-1,3-glucan, is involved in fluconazole resistance in this fungus. Hence, the use of β-1,3-glucanase increases the sensitivity of the biofilm to this drug. It is also suggested that β-1,3-glucans present in the biofilm extracellular

matrix can bind specifically to amphotericin B, thereby offering protection to *Candida* cells [Gulati *et al.* 2016]. Species that are unable to produce β-1,3-glucan are sensitive to fluconazole [Ponde *et al.* 2021]. Our study demonstrated that all the *C. albicans* strains were sensitive to fluconazole, voriconazole, anidulafungin, micafungin, and amphotericin B; in turn, 50% of the strains showed resistance to itraconazole and 16.6% were resistant to posaconazole. Since mixed biofilms can be detected in clinical practice, the presence of other *Candida* species in the oral cavity (*C. glabrata*, *C. krusei*, *C. dublinensis*) may affect on effectiveness of elimination such biofilms.

Analysis of *C. albicans* biofilm in light, SEM, and AFM microscopy

As specified in the aim of the study, only *C. albicans* strains were used in further analyses. In laboratory conditions, biofilm formation on a glass substrate was induced for 24 h. The observations of *C. albicans* carried out in 24-h cultures with the use of the light microscope revealed a tendency of the cells to form a biofilm structure with multilayer cell systems. The SEM visualizations showed the formation of biofilm scaffolding and a network of cells and pseudohyphae (Fig. 1/1A-1D). The diameter

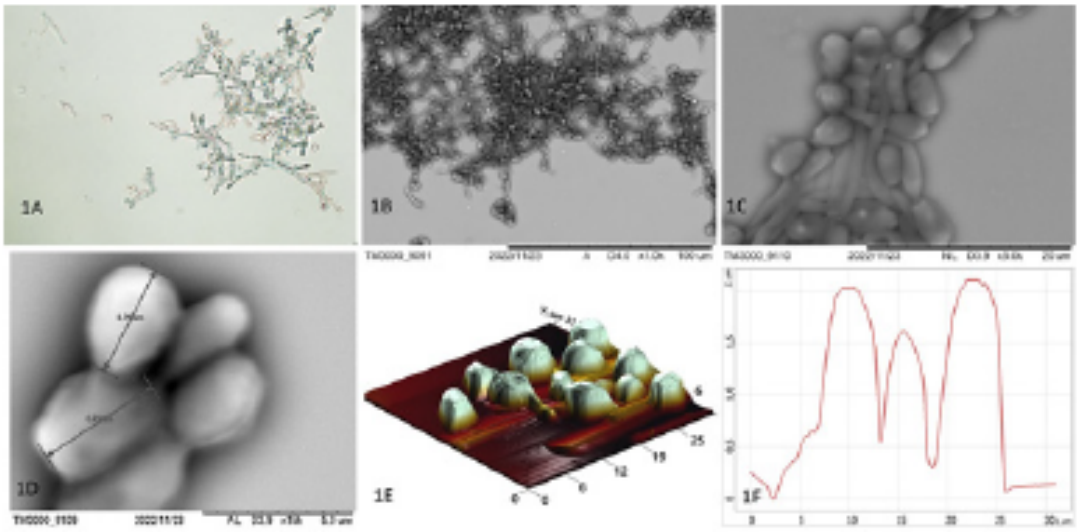

Fig. 1. C. albicans biofilm formation on glass plates in 24-h liquid cultures.

of *C. albicans* cells in this structure was 5.75 µm (min. 3.72 µm, max. 7.73 µm, SD 0.66), and the length of the pseudohyphae was 17.08 µm (min. 6.88 µm, max. 40.40 µm, SD 6.85). The diameters measured with the use of AFM (mean value 4.56 µm, SD 0.84, min. 2.73, max. 6.61) were similar to those obtained in SEM. The height of the cells was measured using the Height Signal in AFM; its average value was 2.84 µm (SD 0.29, min. 1.98 µm, max. 3.35 µm) (Fig. 1/1E-1F; Fig. 2/2A-2B); similar values were reported by El-Baz (2.33 µm) [El-Baz *et al.* 2021] and Tyagi (2.00 µm) [Tyagi *et al.* 2010]. Additionally, the presence of an extracellular substance surrounding the *C. albicans* cells was detected. Figure 2 shows a cell in this structure with a height in the range of 70-200 nm (Fig. 2/2C-2D).

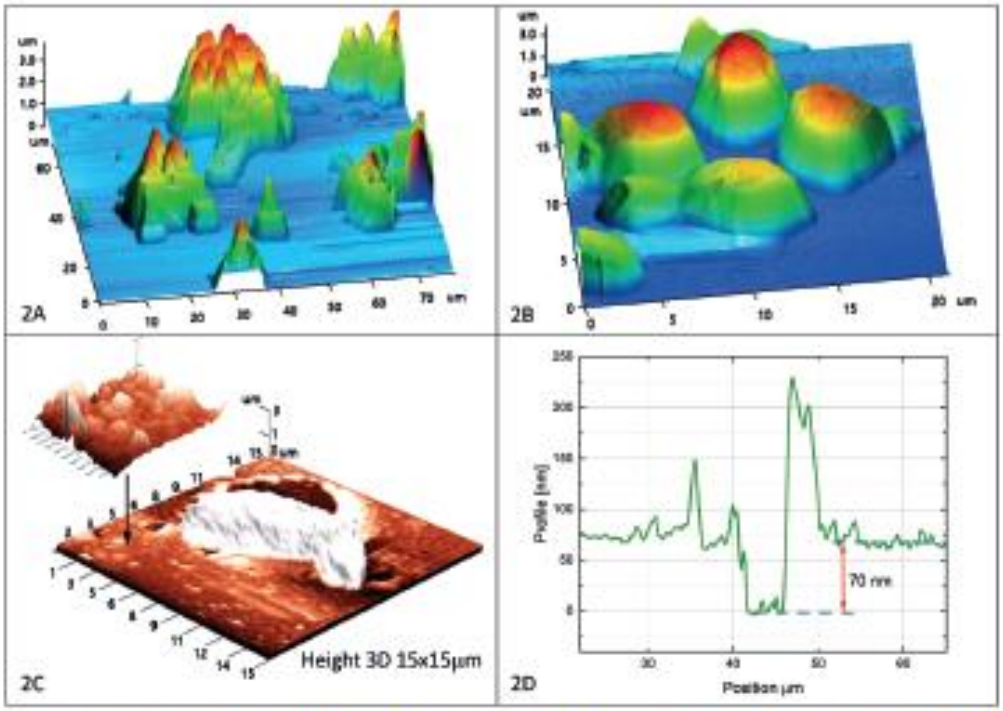

Fig. 2. Topography of C. albicans cells in 24-h cultures on glass with a visible extracellular substance.

The biofilm formation process is influenced by the surface type and environmental factors. The formation of a mature biofilm was observed after 24-48 h on polyvinyl chloride surfaces and after 38-72 h on polymethyl methacrylate surfaces [Ponde *et al.* 2021]. In the present study, the yeast cells were found to adhere to the glass surface after 24 hours and the presence of microcolonies as well as hyphae stabilizing of the structures was detected. Compact, multi-layered cell arrangements were observed in SEM. The biofilm formation ability and virulence of *C. albicans* are associated with the transition from the yeast to hyphal morphotype. Hyphae stabilize the biofilm and have an impact on its layered structure in mature forms, whereas the basal layer plays an important role in the adhesion of the biofilm to the surface. Dispersion of yeast-like cells from the mature biofilm formed on biotic surfaces to other areas in the organism has been reported [Sztajer *et al.* 2014, Gulati *et al.* 2016, Lohse *et al.* 2018, Nobile *et al.* 2015, Ruszkowski *et al.* 2020, Ponde *et al.* 2021]. Therefore, the ability of *C. albicans* to form hyphae is important for the development and maintenance of the biofilm, and strains that do not produce hyphae have an impaired ability to form biofilms [Ponde *et al.* 2021]. The early stage of biofilm formation is influenced by electrostatic interactions determining the process of cell adhesion to biotic or abiotic substrates, van der Waals forces, Brownian movement forces, and hydrophobic interactions. A number of adhesins, e.g. the agglutinin-like sequence (Als) family, the hyphal wall protein (Hwp) family, and others, are expressed in the process. They contribute to effective adhesion of fungal cells to the surface, thereby ensuring biofilm stability [Ponde *et al.* 2021].

AFM analyses of pathogenic microorganisms, including fungi, can be performed using air-dry and liquid preparations. The latter method allows visualization of cells in an environment similar to their natural growth conditions. We employed this type of AFM analysis in the present study. In the liquid environment, the *C. albicans* cell wall is smoother and does not exhibit changes that might be induced by drying, and the cells retain their natural turgor and elasticity [Füzik *et al.* 2015].

The AFM Error Signal (Fig. 3/3B) provides additional qualitative information about the topography of the viewed sample. It facilitates discrimination of small elements against the background of large, relatively smooth surfaces. Areas with different friction coefficients were observed (Lateral Force, LF signal) in the analyzed *C. albicans* samples (Fig. 3/3C). Bright areas with greater friction (rougher) were visible at the sites of contact between the cells and the glass substrate. No bright zones were visible in the lateral and apical cell areas, which proved their lower roughness (greater smoothness). Areas with increased hardness (less elastic; FMM signal) were found mainly in the zones of cells in contact with the glass substrate. The apical areas of the cells, visible as darker zones, were characterized by greater surface elasticity (Fig. 3/3D).

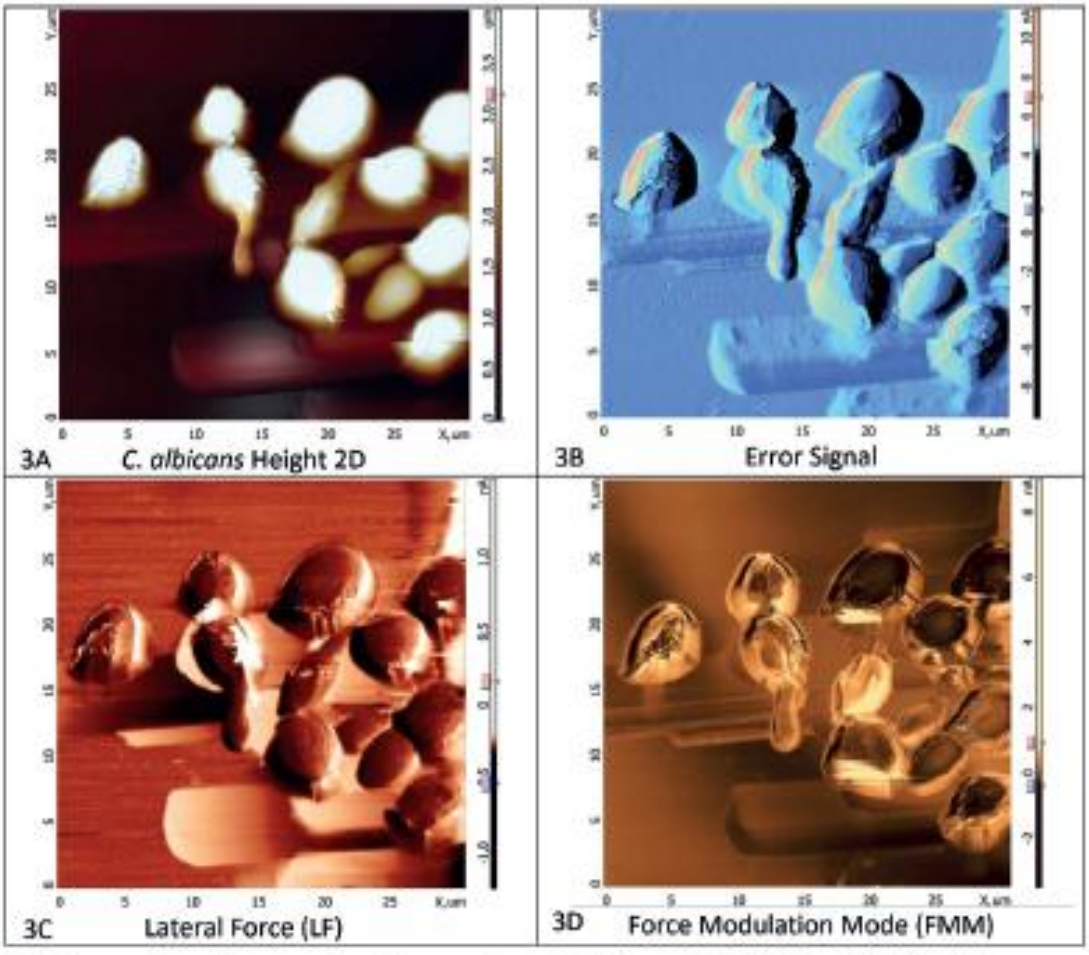

Fig. 3. Topography and nanomechanical properties of C. albicans visualized by AFM (liquid).

Changes in the natural roughness of the surface of *C. albicans* cells and a decrease in their height were observed in AFM upon treatment with essential oils exhibiting antifungal properties (cinnamon oil - MIC 64-500 µg/ml, clove oil - MIC 64-2000 µg/ml, jasmine and rosemary oils - MIC 16-2000 µg/ml). The exposure to clove, cinnamon, jasmine, and rosemary oils resulted in a decrease in the cell height to 381±80 nm, 438±80 nm, 117±25 nm, and 1287±190 nm, respectively, versus the height of 2332±450 nm of control cells. The study conducted by El-Baz also showed that the adhesive capacity of cells increased with the increasing roughness of their surface [El-Baz *et al.* 2021].

The adhesion forces (F_a) determined for the *C. albicans* cells had the same order of magnitude in each case, while the shape of individual curves differed from each other. This suggests gradual detachment of the microscope tip from the sample (cell) surface and indicates "individual" features of the surface of cells of the biofilm-

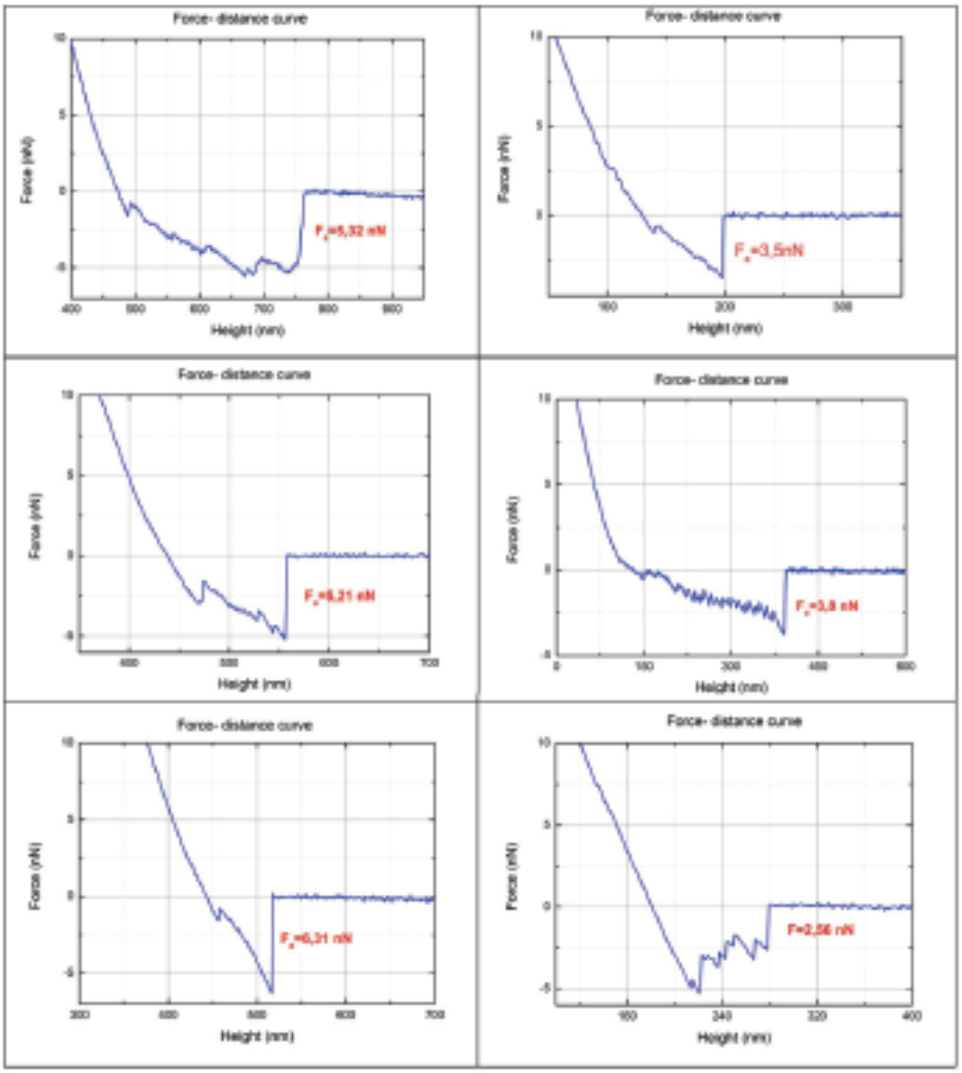

Fig. 4. Adhesion forces (Fa) determined for biofilm-forming C. albicans cells in 24-h cultures.

forming microcolonies (Fig. 4). The mean value of the adhesion force ($\bar{F}_a$) determined for *C. albicans* cells present in the biofilm structure after 24-h culture was 4.01 ± 1.64 nN (min. 2.56 nN, max. 6.3 nN). The nanomechanical properties of cells change under the influence of antifungal substances, although they may also be associated with the characteristics of a given strain. Wild-type *Candida* cells were reported to have greater surface elasticity than cells of two mutants (*cho1* Δ/Δ; *kre5* Δ/Δ) or caspofungin-treated cells [Hasim *et al.* 2017]. The adhesion force (force sufficient to detach the AFM tip from the cell surface) may indicate biofilm stability and damage to the biofilm caused by such degrading factors as antifungal agents. The average value of the adhesion force determined in the present study for *C. albicans* cells forming the biofilm structure in the 24-h cultures was 4.01±1.64 nN. In a study conducted by Ma et al., the adhesion force of *C. albicans* cells was 7.35±0.77 nN; after *Streptococcus sanguinis* bacteriocin treatment, it decreased to 5.69±0.62 nN [Ma *et al.* 2017].

In air-dry preparations analyzed in AFM, it is not possible to visualize the extracellular matrix that binds cells constituting the biofilm. This structure, which is extremely important for biofilm stability, can only be observed in liquid samples. The presence of the extracellular matrix around the *C. albicans* cells was shown by AFM in the 24-h cultures. The extracellular matrix visible in AFM binds neighboring cells, and its direct contact with the biofilm substrate has an impact on adhesion forces. Our observations are consistent with other published data showing that extracellular polysaccharides (EPS) are involved in processes of cell adhesion to the surface where biofilm structures are formed and ensure biofilm stability [Algburi *et al.* 2017, Le *et al.* 2022]. An AFM study conducted by Lal et al. demonstrated the presence of EPS in *C. albicans* biofilms [Lal *et al.* 2008]. It has been proved that EPS in planktonic *C. albicans* cells contains mainly carbohydrates, proteins, hexosamine, and phosphorus, while the EPS composition in *C. albicans* biofilm structures has significantly lower amounts of carbohydrates and proteins. The differences in the composition of matrix EPS exert an effect on the process of diffusion of antifungal drugs into the biofilm structure [Lal *et al.* 2008]. The extracellular matrix protects microorganisms in single-species and mixed biofilms against mechanical stresses, effects of environmental factors, and elements of the host immune defense [Ponde *et al.* 2021]. The presence of glucans in the extracellular matrix may inhibit the activation of neutrophils, and thus protect *Candida* cells against host defense mechanisms. In turn, hyphae are able to damage phagocytic cells mechanically and penetrate epithelia during invasion [Nobile *et al.* 2015, Gulati *et al.* 2016]. Natural products can also interact with biofilms. The study conducted by Ansari et al. in AFM and SEM showed that Jujube (*Zizyphus spina-christi)* honey affects biofilms by decreasing the size of mature biofilms and by disruption of their structure. This type of honey affected the cellular morphology of *C. albicans* [Ansari *et al.* 2013]. The antimicrobial peptide (AMP) Psd1 isolated from *Pisum sativum* seeds degraded biofilms and planktonic cell *C. albicans*. Surface alterations, with membrane disruption and leakage of cellular contents, were observed [Gonçalves *et al.* 2017].

Candida spp. infections may also affect warm-blooded animals, e.g. poultry, horses, cows, dogs, and cats. *C. albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis* as well as *C. guilliermondii* and other [Seyedmousavi *et al.* 2018, Różański *et al.* 2019]. *Candida* spp. species are isolated from these animals most often. Many of these species are potentially pathogenic to humans, especially immunocompromised patients, the elderly, and subjects with chronic health problems. The presence of *Candida* spp. especially in companion animals (dogs, cats) poses a risk of their transmission to the human organism. Additionally, the increased use of antifungals in susceptible patients may lead to a higher incidence of candidiasis, especially caused by rare species (e.g. *Candida bovina*) [Brunet *et al.* 2020]. For this reason, it is advisable to undertake additional research to analyze the ability of zoonotic-origin *Candida* spp. to form biofilms with comparison of the biofilm-formation capacity of species isolated from humans.

Conclusions

In conclusion, yeasts as opportunistic pathogens can cause endogenous infections in certain conditions. The first stage involves colonization of the surface and biofilm formation, and the key colonization processes take place within 24 h. This is evidenced by the arrangement of cells and pseudohyphae in compact structures visible in SEM. The extracellular matrix visualized by AFM binds neighboring cells, and its direct contact with the biofilm substrate has an impact on adhesion forces. The nanomechanical properties of cell walls, e.g. stiffness, roughness and, consequently, susceptibility to deformation and perforation, as well as adhesion forces may have an impact on the quality of the biofilm.

The analysis of *Candida* cells in liquid samples gives satisfactory results, as it prevents unfavorable changes in the cell surface and thus provides more reliable results. The quality of the biofilm is probably related to the nanomechanical properties of *C. albicans* cells and may consequently contribute to the stability of the biofilm structures and their susceptibility or resistance to antifungal drugs. This requires further analyses on a larger number of strains with comparison of such parameters as elasticity, roughness, and adhesion force with consideration of the impact of antifungal substances on the biofilm formation process and on mature biofilms.

Conflict of interest

The authors declare no conflicts of interest.

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22.07.2025 Białe Podlaskie

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Małgorzata Tokarska - Rodak

Imię i nazwisko

Zakład Zdrowia Publicznego Akademii Bialskiej im. Jana Pawła II

Afiliacja

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Oświadczam, że w pracy:

Pańczuk A, Tokarska – Rodak M, Teodorowicz P. Prevalence of Borrelia burgdorferi and Anaplasma phagocytophilum in Ixodes ricinus collected from dogs in easter Poland. 2024. Journal of Veterinary Research 68.1: 109. DOI: 10.2478/jvetres-2024-0015

mój udział polegał na:

analizie i interpretacji danych, przygotowaniu artykułu, krytycznej rewizji i opracowaniu finalnej wersji artykułu (34% wkładu)

Małgorzata Tokarska-Rodak

Podpis

22.07.2025, Biała Podlaska

Data i miejsce

Patrycja Andrzejuk

Imię i nazwisko

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Patrycja Andrzejuk

Podpis

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J Vet Res 68, 109-114, 2024
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Prevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in *Ixodes ricinus* collected from dogs in eastern Poland

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Abstract

Introduction: *Ixodes ricinus* ticks are an important vector and reservoir of pathogenic microorganisms causing dangerous infectious diseases in humans and animals. The presence of ticks in urban greenery is a particularly important public health concern due to the potential for humans and companion animals to be exposed to tick-borne diseases there. The study assessed the prevalence of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* infection in *I. ricinus* ticks feeding on dogs. **Material and Methods:** The study consisted in analyses of *I. ricinus* ticks collected in 2018–2020 from owned and stray dogs in the north-eastern part of Lubelskie province (eastern Poland). An AmpliSens PCR kit was used for qualitative detection and differentiation of tick-borne infections. **Results:** Infections of *B. burgdorferi* and *A. phagocytophilum* were detected in 10.9% and 12.9% of the examined ticks, respectively. One tick (0.7%) was co-infected by both pathogens. Infection with *B. burgdorferi* was significantly more highly prevalent in ticks collected from the owned dogs than from the strays (18.7% and 2.8%, respectively), whereas the prevalence of *A. phagocytophilum* was similar in both groups (12.0% and 13.9%, respectively). **Conclusion:** The co-infection observed in the study suggests the possibility of simultaneous infection by both pathogens from a single tick bite. The presence of pathogens in ticks collected from dogs is a factor in assessing infection risk not only to companion animals but also to their owners, who are in close contact with their dogs and visit the same green areas recreationally.

Keywords: ticks, tick-borne infections, canine borreliosis, anaplasmosis, eastern Poland.

Introduction

Ixodes ricinus is the most widespread tick species in Europe. Its geographic range has expanded considerably in recent decades. The species can now be found in areas and habitats located further north and at higher altitudes than several decades ago (5, 10, 12). These ticks prefer habitats with deciduous and mixed (or less often coniferous) forests with a thick litter layer. As a result of changes in land use and wildlife management, ticks can also be found in urban and suburban areas in many European countries (9, 19, 24, 26, 36). In urban habitats, small, medium-sized and larger mammals (roe deer and wild boar), birds, and companion animals (dogs and cats) contribute to the maintenance of tick populations and are reservoirs of tick-borne pathogens. The presence of ticks in urban greenery is of particular concern in respect of public health, due to the potential exposure of visiting humans and companion animals to tick-borne diseases (26) and the commonality of major tick-borne infectious diseases to humans, dogs and cats. *Ixodes ricinus* is one of the most important vectors and reservoirs of the pathogens which cause dangerous infectious diseases in humans and animals (11). Borreliosis, anaplasmosis, ehrlichiosis and rickettsiosis are the most important bacterial diseases transmitted by *I. ricinus* (30), the first of these being the most common vector-borne disease in the northern hemisphere (16). Spirochetes from the complex *Borrelia burgdorferi* sensu lato (s.l.) transmitted by ticks are the aetiological agents of the disease. Although most dogs exposed to *Borrelia* infections remain clinically asymptomatic (4, 18), cases of clinical canine borreliosis have been reported in almost all European countries, Poland being no exception (17). Canine borreliosis most often has an arthritic form, manifesting as inflammation in the extremities, usually of the carpal or tarsal joints. These symptoms are accompanied by malaise (fever, lack of appetite and fatigue) and

lameness developing after a few days. Myocarditis is rarely diagnosed in canine borreliosis; however, the renal form of the disease and neurological dysfunctions may appear in older dogs (29). *Anaplasma phagocytophilum* is the aetiological agent of human granulocytic anaplasmosis (1). Granulocytic anaplasmosis has been diagnosed in various species of wild and domestic animals, including dogs (34). The clinical spectrum of the disease ranges from subclinical and self-limiting to subacute, chronic or severe disease in immunocompromised patients. The clinical signs of the disease vary in severity but are usually non-specific, *e.g.* fever, lethargy and anorexia (2), the first two of these being the most common clinical signs in infected dogs, and ones appearing after an incubation period of 1–2 weeks (3). Most dogs naturally infected by *A. phagocytophilum* will probably remain healthy (3). The concomitant occurrence of anti-*A. phagocytophilum* and anti-*B. burgdorferi* antibodies was observed in two healthy dogs (2/100) from the Lubelskie province and two healthy dogs (2/100) from the Mazowieckie province (Poland) (6). Since *A. phagocytophilum* is transmitted by the same *Ixodes* species as *B. burgdorferi* and is maintained in sylvatic cycles with the same rodent reservoirs, co-infections by these pathogens are possible, which may result in mutual enhancement of the pathogenicity of these microorganisms (21).

The study conducted by Dzięgiel *et al.* (6) in 400 healthy dogs showed that tick control was important as a protective factor against *A. phagocytophilum* and *B. burgdorferi*, while the breed (pure) was a risk factor for *B. burgdorferi* infection. In Europe, the prevalence of *B. burgdorferi* spirochetes in *I. ricinus* ticks varies considerably. A meta-analysis of European studies from 1984–2003 revealed a 13.7% prevalence of *B. burgdorferi* s.l. (25). A later pan-European meta-analysis based on data published in 2010–2016 showed the presence of *B. burgdorferi* s.l. in 14,134 (12.3%) of the 115,028 examined ticks (33). In terms of European regions, the highest *B. burgdorferi* prevalence was found in Central Europe (19.3%). In this region, the data for the meta-analysis were provided by Austria, the Czech Republic, Germany, Hungary, Slovakia, Switzerland and Poland (33). While the meta-analyses did not reveal an upward trend in the prevalence of *B. burgdorferi* infection in *I. ricinus* ticks (25, 33), this does not exclude possible changes in the prevalence in individual regions. A study of *I. ricinus* ticks collected from vegetation in Lublin province (eastern Poland), the north-eastern part of which was the research area in the present study, reported a significant increase in *B. burgdorferi* s.l. infection rates. Two periods separated by a five-year interval were analysed in that study. The incidence of *B. burgdorferi* s.l. infection was 6.0% in 2008–2009 and 15.3% in 2013–2014 (37). The increase in the prevalence observed in the Lublin region most likely has a focal character. As emphasised by the authors, the prevalence of tick infections recorded at the end of the study is similar to the European mean value and is not alarming, but the high rate of the increase in this parameter may indicate a potential risk. One of the possible causes of the rise in the infection rate suggested by the authors is the growing recreational activity in the analysed area and the presence there of greater numbers of humans and companion animals, *i.e.* potential tick hosts (37). Given the great human and veterinary medical importance of the *I. ricinus* tick problem, the ever-higher abundance of these ticks in urban and suburban areas, and the close contact between humans and dogs, it was regarded as worthwhile to make an attempt to assess the prevalence of infection of *I. ricinus* ticks feeding on dogs with *B. burgdorferi* s.l. and *A. phagocytophilum*.

Material and Methods

Study area. The study was carried out in Biała Podlaska county (51°58′20″N, 23°9′12″E) located in the north-eastern part of Lubelskie province. The northern and north-eastern regions of Lubelskie are characterised by the highest percentage of grassland cover in the entire region. A large percentage of the land in this area is also fallows, wasteland and island forests (38). In 2018, the forest cover in Biała Podlaska county was 27.6%, compared with 23.4% in Lubelskie province as a whole (32). Ticks were collected from host or carrier dogs in Łęgi (52°10′03″N, 23°28′11″E), Biała Podlaska (52°01′56″N, 23°06′59″E), Porosiuki (52°01′00″N, 23°03′28″E), Mokre (51°51′01″N, 23°04′38″E) and Janówka (51°58′08″N, 23°04′56″E).

Tick collection. The study involved 147 adult *Ixodes ricinus* ticks (81 females and 66 males) collected from dogs in 2018–2020. Ticks attached to the skin or present on the coat of the dogs were collected once a year. Almost half of the specimens, 72 ticks (49.0%), were collected from 33 homeless dogs kept in a shelter. The other 75 ticks (51.0%) were collected from 7 owned dogs. Ticks were collected twice from two owned dogs and six times from one owned dog after a walk in different locations in the study area, none of which were engorged. Among the 147 collected ticks, 33 (22.4%) specimens were engorged: 30 of them from 21 shelter dogs (3 or fewer engorged ticks per dog) and 3 from 2 owned dogs (with no recurrent infestation). The ticks were collected by the dogs' owners and delivered to the laboratory. The tick species, sex and developmental stage were identified in accordance with the key developed by Nowak-Chmura (23). The ticks were kept individually in Eppendorf tubes in 70% ethanol at 6°C.

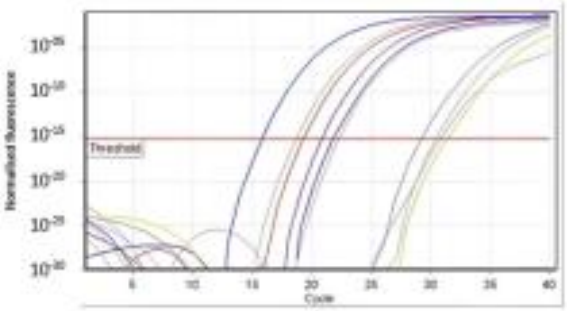
DNA analysis. AmpliSens TBEV, *B.burgdorferi* s.l., *A.phagocytophilum*, *E.chaffeensis*/*E.muris*-FRT (Ecoli Dx, Bratislava, Slovak Republic) for specific detection of a fragment of the 16S ribosomal RNA gene in *B. burgdorferi* s.l. and a fragment of the merozoite surface protein 2 gene in *A. phagocytophilum* was used in the study. The analyses consisted of several steps. The first step was based on the use of the RIBO-prep kit (Ecoli Dx, Bratislava, Slovak Republic) for isolation of genetic material from tick tissues. The next step involved the reverse transcription reaction, which allowed the

synthesis of complementary DNA (cDNA) with the use of the REVERTA-L reagent kit (Ecoli Dx, Bratislava, Slovak Republic). The samples prepared in this way were directly subjected to a real-time PCR reaction using the TBEV, *B. burgdorferi* s.l., *A. phagocytophilum*, *Ehrlichia chaffeensis*/*E. muris*-FRT PCR kit (Ecoli Dx, Bratislava, Slovak Republic), which contained the following reagents: PCR-mix-1-FRT tick-borne encephalitis virus (TBEV), *A. phagocytophilum*, *E. chaffeensis*/*E. muris*, PCR-mix-1-FRT *B. burgdorferi* s.l./internal control (IC), RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), cDNA TBEV, *B. burgdorferi* s.l., *A. phagocytophilum*, *E. chaffeensis*/*E. muris* positive control, DNA buffer and IC. The real-time PCR reaction was performed in a Rotor Gene Q 2 Plex HRM thermal cycler (Qiagen, Hilden, Germany). The specificity of the apparatus, which is equipped with two fluorescence detection channels, was sufficient for identifying a smaller number of tick-borne pathogens than that facilitated by the kit. The reaction also included three checkpoints for each fluorescence channel: a positive control of amplification, a negative control of extraction (C−), and a negative control of amplification. The following amplification procedure was used: 1 cycle at 95°C for 15 min; 5 consecutive cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 15 s; and 40 consecutive cycles at 95°C for 10 s, 56°C for 30 s and 72°C for 15 s. The test used in this study has high specificity confirmed in laboratory clinical studies. The primers and probes were checked for potential homology to all sequences deposited in GenBank using comparative sequence analysis.

The χ^2 test was used to test the significance of differences in the number of infected and non-infected ticks between the different groups. The statistical analysis was performed in the STATISTICA 13.0 program (StatSoft) at a significance level of P-value < 0.05.

Results

Borrelia burgdorferi s.l. infection was detected in 10.9% (16/147) and *A. phagocytophilum* in 12.9% (19/147) of the *I. ricinus* ticks (Fig. 1). One male tick (0.7%) was co-infected by both pathogens and was a specimen collected from an owned dog after a walk in Łęgi. Infection by at least one of the pathogens was detected in 23.1% (34/147) of the ticks (Table 1). The presence of *B. burgdorferi* was detected significantly more frequently (P-value = 0.0103) in male ticks (18.2%) than in females (4.9%). The prevalence of *A. phagocytophilum* infection in the male and female specimens was similar, with a 1.5% greater prevalence in females (13.6% compared with 12.1%). Most of the ticks were not engorged at the time of collection (29/34). Each engorged tick (5/34) was collected from a different homeless dog kept in the shelter. Of those five engorged ticks, one was infected by *B. burgdorferi* and four by *A. phagocytophilum*. *Borrelia burgdorferi* infection was significantly more frequent (P-value = 0.0020) in ticks collected from the owned dogs (18.7%) than from the shelter dogs (2.8%). In contrast, the prevalence of *A. phagocytophilum* infection was similar in both groups (12.0% and 13.9%, respectively).



No.	Colour	Sample	Pathogen	Ct
1		1101	*A.ph*	30.76
2		C-		
3		NCA		
4		C+		21.79
5		228	*B.b.sl*	18.64
6		229	*B.b.sl*	29.18
7		231	*B.b.sl*	19.26
8		232	*B.b.sl*	31.30
9		233	*B.b.sl*	15.74
10		234	*B.b.sl*	20.82
11		C--		
12		NCAII		
13		C++		22.21

Fig. 1. Examples of PCR results positive for complimentary DNA of *Anaplasma phagocytophilum* (*A.ph.*) and *Borrelia burgdorferi* sensu lato (*B.b.* s.l.). C- – negative extraction control; NCA – negative amplification control; C+ – positive amplification control; C– – positive amplification control and internal control; NCAII – negative amplification control and internal control; C++ – positive amplification control and internal control; Ct – threshold cycle

Table 1. *Borrelia burgdorferi* sensu lato (s.l.) and *Anaplasma phagocytophilum* infection in *Ixodes ricinus* ticks collected from dogs in the north-eastern part of Lubelskie province, eastern Poland

		Total number (%) of collected ticks	Number (%) of positive ticks for each pathogen		Total number (%) of infected ticks
			B. burgdorferi s.l.	*A. phagocytophilum*	
Ticks	Female	81 (55.1)	4 (4.9)	11 (13.6)	15 (18.5)
	Male	66 (44.9)	12 (18.2)	8 (12.1)	19 (28.8)*
	Engorged	33 (22.4)	1 (3.0)	4 (12.1)	5 (15.2)
	Non-engorged	114 (77.6)	15 (13.2)	15 (13.2)	29 (25.4)*
Dogs	Stray	72 (49.0)	2 (2.8)	10 (13.9)	12 (16.7)
	Owned	75 (51.0)	14 (18.7)	9 (12.0)	22 (29.3)*
Total		147 (100.0)	16 (10.9)	19 (12.9)	34 (23.1)*

* – the infected tick total is lower by one than the sum of the per-pathogen infected tick numbers because *A. phagocytophilum* and *B. burgdorferi* s.l. were present simultaneously in one of the ticks

Discussion

Companion animals are more susceptible to contact with ticks than humans are, as they tend to spend more time outdoors at a closer distance to the ground and vegetation and have a coat which ticks attach to easily (31). Investigations of the prevalence of pathogen infection in ticks infesting domestic animals allow assessment of the risk of tick-borne diseases in these animals and of the potential risk of infection posed to animal owners. Dogs can carry infected ticks from the natural environment to human habitats, as dogs and humans share the same living space and visit the same outdoor areas (7). Studies on *I. ricinus* ticks collected from domestic animals in different European countries showed a varied prevalence of *B. burgdorferi* and *A. phagocytophilum* infections. A higher percentage of infections with *B. burgdorferi* than with A. *phagocytophilum* was reported from Germany (11.6% and 6.5%, respectively) (28) and the Netherlands (7.2% and 1.6%, respectively) (22). In contrast, a higher prevalence of *A. phagocytophilum* infection was observed in ticks collected from dogs and cats in Belgium. The presence of *A. phagocytophilum* and *B. burgdorferi* was detected in 19.0% (127/668) and 11.1% (83/745) of *I. ricinus* ticks, respectively (4). A higher prevalence of *A. phagocytophilum* was also reported in studies conducted by Geurden *et al.* (8) on *I. ricinus* ticks collected from dogs in Hungary, France, Belgium and Italy, where the 15% of ticks infected by these bacteria was remarkably higher than the 1% of ticks infected by *B. burgdorferi*. Among the analysed countries, Hungary alone provided an instance of *B. burgdorferi* infection. This country also had the highest level of *A. phagocytophilum* infection in ticks at 20%, France with a 14% rate having the next highest. The pathogen was not detected in ticks collected in Belgium or Italy (8). The low prevalence or absence of infections reported in these studies, especially in the case of *B. burgdorferi*, may be associated with the small number of ticks analysed in each of these countries.

Within Poland, the presence of *B. burgdorferi* spirochetes in *I. ricinus* collected from dogs also varied greatly. It ranged from 0%, as shown in studies on domestic dogs from Zakopane in southern Poland (the Polish town at the highest altitude) (13) to 35.7% reported in studies on dogs admitted to veterinary clinics in the city of Olsztyn in north-eastern Poland (20). The prevalence of these pathogens was estimated at 6.2% in studies conducted in the Warsaw agglomeration in central Poland (39) and 22.5% in analyses of ticks collected from dogs and cats in the Wrocław agglomeration in south-western Poland (14). These investigations did not reveal statistically significant differences in the infection prevalence between ticks collected from dogs and those collected from cats.

In the present study of ticks collected from dogs in the north-eastern part of Lubelskie province in eastern Poland, *B. burgdorferi* infection was detected in 10.9% (16/147) of the analysed specimens. This percentage is very similar to the results reported in a study on ticks collected from domestic animals in several veterinary clinics in Lublin in the central part of the province, where 11.8% (16/136) of *I. ricinus* ticks collected from dogs and cats were infected by *B. burgdorferi* (27). The *A. phagocytophilum* infection rate was 12.9% (19/147). Male ticks had *B. burgdorferi* infection significantly more frequently (P-value = 0.0103) than females (18.2% compared with 4.9%). The prevalence of *A. phagocytophilum* infection in the male and female specimens was similar, males being slightly less frequently infected (12.1% compared with 13.6%). In total, infection by at least one of the pathogens was detected in 23.1% (34/147) of the ticks (Table 1). *Borrelia burgdorferi* infection was significantly more frequent (P-value = 0.0020) in ticks collected from the owned dogs (18.7%) than from the homeless animals (2.8%); however, the prevalence of *A. phagocytophilum* infection was similar in both groups (12.0% and 13.9%, respectively). The prevalence of *A. phagocytophilum* infection in *I. ricinus* ticks collected from dogs or dogs and cats in Poland ranged by region of collection from 1.2% to 21.3% (16), the lowest percentage having been reported in ticks from dogs admitted to veterinary clinics in the city of Olsztyn. Noteworthily, a different study showed the highest rates of *B. burgdorferi* infections in ticks collected from dogs (35.7%) in this region of Poland (20). In turn, the highest prevalence of *A. phagocytophilum* infections (21.3%) was reported in a study of ticks collected from dogs and cats in the Wrocław agglomeration in south-western Poland. It is worth noting that the study did not show statistically significant differences between the pathogen positivity rate in ticks infesting cats and in those infecting dogs (15). Low *A. phagocytophilum* infection rates were reported in other studies conducted in Poland. *Anaplasma phagocytophilum* DNA was detected in 2.9% of *I. ricinus* ticks collected from dogs in the Warsaw agglomeration and in 3.4% of ticks infesting domestic dogs and cats from Zakopane in southern Poland (13). In the present analyses of ticks collected from dogs in the north-eastern part of Lubelskie province, 12.9% of the specimens were infected by *A. phagocytophilum*. This result was higher than the value reported from the central part of the province, which showed *A. phagocytophilum* infection in 8.8% (12/136) of *I. ricinus* ticks collected from dogs and cats in several veterinary clinics in Lublin (27). This finding was an interesting difference from the near-parity of *B. burgdorferi* infection rates across the province.

Ticks can serve as a reservoir and vector of more than one pathogen. Co-infections are becoming an increasingly serious clinical problem and a more needful research area because their ecology and pathological mechanisms are still poorly explored in comparison with single-pathogen infections. The best-known co-infection in ticks and humans is that caused by the pathogens of current interest, *A. phagocytophilum* and *B. burgdorferi* s.l. (21, 35). Previous studies in Poland rarely reported the presence of this co-infection in *I. ricinus* ticks infesting

domestic animals. No such co-infection was reported in studies from north-eastern (20) or southern Poland (13). However, the present study focused on eastern Poland revealed this co-infection in one *I. ricinus* tick (0.7%). This low prevalence contrasts sharply with the high prevalence of the co-infection reported in a study conducted by Roczeń-Karczmarz *et al.* (27) in south-eastern Poland, *i.e.* a region neighbouring the present study area. The authors examined ticks collected from vegetation and infesting dogs and cats. Co-infection with *A. phagocytophilum* and *B. burgdorferi* was detected in 14.0% of the analysed *I. ricinus* specimens. These results indicate a high risk of simultaneous transmission of both pathogens through a single tick bite and concurrent development of Lyme disease and anaplasmosis.

Conclusion

The results of the present study confirm the presence of *B. burgdorferi* and *A. phagocytophilum* in *I. ricinus* ticks infesting dogs in eastern Poland and indicate the prevalence of infection caused by these pathogens to be similar. The co-infection detected in an examined tick suggests the possibility of simultaneous infection by both pathogens through a single tick bite. With the knowledge of the presence of *B. burgdorferi* and *A. phagocytophilum* in ticks collected from dogs, more accurate assessment is possible of the risk of infection posed to companion animals, and by extension to their owners who are in close contact with their pets and visit the same green areas recreationally.

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Tick-borne pathogens in *Dermacentor reticulatus* collected from dogs in eastern Poland

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Abstract

In recent years, the distribution of *Dermacentor reticulatus* ticks has expanded into new territories in many European countries, including Poland, with increased population densities in areas of their regular occurrence. The spread of *D. reticulatus* enhances the risk of exposure of domestic animals and their owners to tick-borne diseases. The objective of this study was to assess the prevalence of infection of *D. reticulatus* ticks feeding on dogs with the pathogens *Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum*. The study material comprised 152 *D. reticulatus* ticks collected from dogs in the northeastern part of Lublin Province (eastern Poland). A ready-made AmpliSens® TBEV, *B.burgdorferi sl*, *A.phagocytophilum*, *E.chaffeensis*/*E.muris*-FRT PCR kit was used for qualitative detection and differentiation of tick-borne infections. The assessment of the degree of infection of the analyzed ticks with the two pathogens revealed that 9.2% (14/152) of the examined ticks were infected with one of the pathogens. No co-infections with the pathogens were detected in any of the ticks. The highest specific percentage of infections (8.6%, 13/152) was associated with *A. phagocytophilum*. The presence of *B. burgdorferi* s.l. was detected in only one of the examined ticks (0.7%). The spread of *D. reticulatus* to new territories and the increase in population density in areas of their regular occurrence implies the need for further studies of the prevalence of pathogens with medical and veterinary importance in order to assess the risk of tick-borne diseases.

Keywords *Dermacentor reticulatus* · *Borrelia burgdorferi* · *Anaplasma phagocytophilum* · Dogs · Poland

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Introduction

Dermacentor reticulatus (Fabricius) is the second (after *Ixodes ricinus*) most important reservoir and vector of infectious diseases in Europe. In comparison with *I. ricinus*, the role of this species in the risk of infection by transmitted pathogens has insufficiently been recognized (Grochowska et al. 2020).

Dermacentor reticulatus is characterized by a wide host range, a high reproduction rate, a rapid (typically annual) developmental cycle, and high rates of survival in adverse conditions. The high adaptability of *D. reticulatus* has been confirmed by new data on its occurrence range (Földvári et al. 2016). In recent years, the distribution of these ticks has expanded into new territories in many European countries, including Poland, with increased population densities in areas of their regular occurrence Bullová et al. 2009; Dautel et al. 2006; Földvári et al. 2016; Karbowiak 2014; Kiewra and Czułowska 2013; Kubiak et al. 2018; Mierzejewska et al. 2015, 2016; Naminа et al. 2019; Nowak 2011; Paulauskas et al. 2015; Rubel et al. 2016; Sreter et al. 2005; Široký at al. 2011; Zając et al. 2020b).

In contrast to *I. ricinus*, the occurrence range of *D. reticulatus* does not concern the entire area of Poland. Before the 1990s, *D. reticulatus* localities were found mainly in the north-eastern part of the country. The regions between the Vistula River and the western border of the country were considered free from *D. reticulatus*. These areas were part of the gap in the geographical range of *D. reticulatus* in central Europe. It divided the *D. reticulatus* population into the western and eastern macroregions. However, an expansion of *D. reticulatus* has been observed in Poland for over 2 decades. Currently, the eastern population of this species covers areas from the eastern border of the country to central Poland and spreads further west. In turn, the western population is expanding eastwards (Kiewra and Czułowska 2013; Król et al. 2016; Kubiak et al. 2018; Mierzejewska et al. 2015a, 2016; Nowak 2011; Opalińska et al. 2016; Zając et al. 2020b). The expansion of *D. reticulatus* is so intense that, in some regions of eastern and central Poland, this species dominates over *I. ricinus* (Mierzejewska et al. 2015b; Pańczuk et al. 2021; Zając et al. 2020a, 2021; Zygner and Wędrychowicz 2006).

Dermacentor reticulatus have a wide host range, as >60 wild and domestic animal species have been identified as hosts for the three active developmental stages (larvae, nymphs, and adults). This tick species is referred to as burrow-questing non-nidicolous, i.e., the larval and nymph stages are associated with the burrows of their hosts. Larvae and nymphs usually ingest blood from the same host, usually a small mammal. Typical hosts for *D. reticulatus* larvae are voles, mice, hedgehogs, shrews, moles, hares, and rabbits (birds are occasional hosts). In turn, in addition to the larval hosts, nymphs feed on weasels, polecats, cervids, goats, and dogs. Adult stages have a wider range of hosts, e.g., a variety of cervid species, wild boars, foxes, wolves, hedgehogs, hares, and rabbits from the wild fauna. Domestic animals, mainly dogs, horses, donkeys, cattle, sheep, goats, and pigs, are equally important and sometimes dominant tick hosts in cities or agricultural areas. Domestic animals are infested almost exclusively by adult ticks. Exceptionally, animals that explore burrows may be attacked by juvenile *D. reticulatus* stages (Földvári et al. 2016; Mierzejewska et al. 2015b; Nowak-Chmura 2013; Paziewska et al. 2010; Pfäffle et al. 2015).

Studies of the species composition of ticks feeding on dogs in Poland report a more frequent presence of *D. reticulatus* than *I. ricinus*. *Dermacentor reticulatus* were reported to dominate (64.6%) among ticks collected from dogs near Warsaw (central Poland) in

2003–2005 (Zygner and Wędrychowicz 2006). Even greater dominance of this species (86.1%) was observed in ticks collected from dogs in the Mazovia and Mazuria regions (central and northern Poland) in 2012–2013 (Mierzejewska et al. 2015b). As shown by an 8-year study (2009–2016) conducted in the urban agglomeration of Olsztyn (northern Poland), although *I. ricinus* (60.1%) dominated over *D. reticulatus* (39.7%), there was a gradual increase in the prevalence of the latter species in the subsequent years of the study (in 2016, *D. reticulatus* accounted for 57.9% of ticks collected from dogs) (Michalski 2019). In a 3-year study (2017–2019) carried out in the northeastern part of Lublin Province (eastern Poland), *D. reticulatus* accounted for 55.5% of all collected specimens (Pańczuk et al. 2021). The high percentage of *D. reticulatus* ticks infesting dogs implies the necessity to investigate the prevalence of pathogens with medical and veterinary importance in order to assess the risk of tick-borne diseases. As both *I. ricinus* and *D. reticulatus* infest dogs in their co-occurrence range, there may be a higher probability of co-infection with several pathogens and, consequently, a more severe or atypical course of diseases complicating the diagnosis and therapy.

Dermacentor reticulatus are involved in the transmission of pathogens with medical and veterinary importance. Undoubtedly, the most important pathogen transmitted by these ticks to animals is the protozoan *Babesia canis* (Földvári et al. 2016), and canine babesiosis is one of the most dangerous infectious diseases of dogs in endemic areas. Besides *B. canis*, genetic material of other pathogens has also been detected in *D. reticulatus*, e.g. bacteria of the genus *Rickettsia*, *A. phagocytophilum*, *Borrelia burgdorferi* s.l., *Francisella tularensis*, or TBEV (Ben and Lozynskyi 2019; Biernat et al. 2014; Bonnet et al. 2013; Dzięgiel et al. 2014; Karbowiak et al. 2014; Mierzejewska et al. 2015a; Naminа et al. 2019; Reye et al. 2013; Roczeń-Karczmarz et al. 2018; Rybářová and Široký 2017; Schreiber et al. 2014; Szczotko et al. 2019; Tomanović et al. 2013; Wójcik-Fatla et al. 2011, 2015; Zając et al. 2017). Two of these tick-borne pathogens, *A. phagocytophilum* and *B. burgdorferi* s.l., have now been reported in dogs in nearly all European countries, including Poland (Krämer et al. 2014). Studies on seroprevalence in European dogs have reported that 3–57% of dogs were carriers of *A. phagocytophilum* (Sainz et al. 2015). In Poland, analyses of 3,094 samples of serum collected from dogs from all 16 Polish provinces showed the presence of anti-*A. phagocytophilum* antibodies in 12.3% of dogs, and the presence of anti-*B. burgdorferi* antibodies in 3.8% of dogs. The study demonstrated nationwide occurrence of *A. phagocytophilum* and *B. burgdorferi* s.l. in the studied population of dogs. The highest percentages of dogs (>20%) infected with *A. phagocytophilum* were reported in Lesser Poland, Silesia and Łódź Provinces (southern and central regions of Poland). For *B. burgdorferi* s.l., the highest prevalence (>10%) was noted in dogs from Łódź Province (central Poland) (Krämer et al. 2014). In Europe, *I. ricinus* is a known vector of *A. phagocytophilum* (Sainz et al. 2015) and *B. burgdorferi* s.l. (Skotarczak 2002), but these pathogens have also been detected in other tick species, e.g., *D. reticulatus* (Ben and Lozynskyi 2019; Bonnet et al. 2013; Dzięgiel et al. 2014; Karbowiak et al. 2014; Michalski et al. 2020; Mierzejewska et al. 2015a; Rar et al. 2005; Reye et al. 2013; Roczeń-Karczmarz et al. 2018; Rybářová and Široký 2017; Szczotko et al. 2019; Zając et al. 2017).

The aim of the study was to assess the prevalence of infection of *D. reticulatus* ticks feeding on dogs with the pathogens *B. burgdorferi* s.l. and *A. phagocytophilum*.

Materials and methods

Study area

The study was carried out in the northeastern part of Lublin Province (eastern Poland). The northern and northeastern regions of Lublin Province are characterized by the highest percentage of grasslands in the entire area. A significant percentage of land in this area is also covered by fallow, wasteland, and forest patches. The mosaic character of the landscape provides *D. reticulatus* populations with favorable conditions. Forest areas are associated with the presence of hosts for adult ticks, whereas rodents, i.e., hosts for juvenile stages, inhabit grasslands and wastelands. The area of Lublin Province is characterized by a high density of *D. reticulatus* populations. As reported by Zając et al. (2020b) in a study conducted in 2019, the mean number of ticks collected in Lublin Province amounted to 96.8 specimens/100 m^2, with the highest density noted in the northern part of the province.

In the present study, dogs were examined as hosts or carriers of ticks mainly in the following localities: Biała Podlaska (52°01'56"N, 23°06'59"E), Janów Podlaski (52°11'38"N, 23°12'43"E), Konstantynów (52°12'28"N, 23°05'07"E), Mokre (51°51'01"N, 23°04'38"E), Łęgi (52°10'03"N, 23°28'11"E), Porosiuki (52°01'00"N, 23°03'28"E), Zakalinki (52°12'55"N, 23°02'55"E), Bereza (51°56'14"N, 22°46'39"E), Małaszewicze (52°01'33"N, 23°31'51"E), Styrzyniec (52°01'23"N, 22°59'35"E), and Janówka (51°58'08"N, 23°04'56"E).

Tick collection

The study material comprised 152 *D. reticulatus* ticks (71 females, 81 males) collected from 55 dogs in 2018–2020. Seventy-eight ticks (51.3%) were collected from stray dogs coming from shelters, whereas the remaining 74 ticks (48.7%) were collected from owned dogs. The ticks were collected on a yearly basis. Maximum nine ticks collected from one dog were used for the analyses (average number of ticks per dog: 2.76). Ticks attached to dog's skin and those present on the coat were collected by dogs' keepers and delivered to the laboratory. All ticks analyzed were non-engorged. The species, sex, and developmental stage were identified based on morphological traits with the use of an identification key (Nowak-Chmura 2013). The ticks were stored individually in Eppendorf tubes in 70% ethanol at 6 °C.

Molecular identification of pathogens

DNA analysis

A ready-made AmpliSens® TBEV, *B.burgdorferi sl*, *A.phagocytophillum*, *E.chaffeensis*/*E. muris*-FRT PCR kit (InterLabService, Russia) was used for qualitative detection and differentiation of tick-borne infections. The target of the PCR reaction was the cDNA of *B. burgdorferi* s.l. and *A. phagocytophilum*. The detection concerned a fragment of the 16 S RNA gene in the case of *B. burgdorferi* s.l. and a fragment of the msp2 gene in *A. phagocytophilum*.

DNA Isolation

DNA was isolated from tick tissues with the use of an AmpliSens RIBO-prep kit. The DNA was stored at 2–8 °C for 24 h or at – 16 °C for a longer time.

DNA amplification

A ready-made kit contained the following reagents: PCR-mix-1-FRT TBEV, *A. ph.*, *E. ch./E.m.*, PCR-mix-1-FRT .*B. b. s.*1./IC, RT-PCR-mix-2-FEP/FRT, polymerase (TaqF), positive control cDNA TBEV, *B. b. s.l.*, *A. ph.*, *E. ch./E. m.*/STI, DNA buffer, and internal control (IC). The Real Time PCR was performed in a Rotor Gene Q 2 Plex HRM thermal cycler. Due to the specificity of the available device, which is equipped with two fluorescence detection channels, the present analyses detected fewer tick-borne pathogens than offered by the kit. Fluorescent signal detection is assigned in the channels for the FAM (Green) and HEX (Yellow) fluorophores respectively for Internal Control (IC) and *B. burgdorferi* s.l. and *A. phagocytophilum*. The reaction also included three checkpoints for each fluorescence channel: positive amplification control (C+), negative control of extraction (C-), and negative control of amplification (NCA). The amplification parameters are shown in Table 1.

Interpretation of results

Results are considered reliable only when the extraction and amplification controls are correct. Negative controls are absent and positive controls take values < 27 Ct value for both pathogens. Clinical samples are considered positive for *A. phagocytophilum*, and *B. burgdorferi* s.l. infection at Ct values < 38 in all detection channels.

The analytical specificity of test AmpliSens® TBEV, *B. burgdorferi s.l.*, *A. phagocytophillum*, *E. chaffeensis*/*E. muris*-FRT PCR kit is ensured by selection of specific primers and probes as well as by selection of strict reaction conditions. The primers and probes were checked for possible homologies to all sequences deposited in gene banks by sequences comparison analysis. The clinical specificity of test was confirmed in laboratory clinical trials and no false-positive results were observed during examination of DNA. Due to the high specificity of the test, we assume the high authenticity of the research results obtained.

Table 1 Real Time PCR amplification parameters

Step	Temperature (°C)	Time	Detection of fluorescence	No. cycles
Hold	95	15 min	-	1
Cycling	95	10 s	-	5
	60	30 s	-	
	72	15 s	-	
Cycling 2	95	10 s	-	40
	56	30 s	FAM/Green, HEX/Yellow	
	72	15 s	-	

Results

The assessment of the degree of infection of the analyzed ticks with the two pathogens (*Borrelia burgdorferii* s.l., *A. phagocytophilum*) revealed that 14 (9.2%) of the 152 examined ticks were infected with one of the pathogens. No co-infections with the pathogens were detected in any of the ticks. The highest percentage of infections was associated with *A. phagocytophilum*. Its presence was detected in 8.6% (13/152) of the examined specimens. The percentage of *A. phagocytophilum* infections was the same in female and male ticks, i.e., 8.5% (6/71) and 8.6% (7/81), respectively. The ticks were collected from nine dogs. Nine ticks were from owned dogs (one tick each from three dogs, two ticks each from another three dogs) and the remaining four ticks were from stray dogs from a shelter (one tick each from two dogs, two ticks from one dog). Apart from the 13 *D. reticulatus* ticks infected with *A. phagocytophilum*, no other tick species was found on the dogs at the time of collection. The presence of *B. burgdorferi* s.l. was detected in only one of the examined ticks (0.7%). This tick was collected from a dog that came from a shelter (Table 2). Two *I. ricinus* ticks were also collected from this dog. Studies revealed that *B. burgdorferi* s.l. and *A. phagocytophilum* were not detected in any of these ticks.

Discussion

Anaplasma phagocytophilum

Anaplasma phagocytophilum is the causative agent of human granulocytic anaplasmosis (HGA) (Bakken and Dumler 2015). The disease has been diagnosed in various species of wild and domestic animals, including dogs (Stuen et al. 2013). In Europe, *I. ricinus* is a known vector of *A. phagocytophilum* (Sainz et al. 2015), but this pathogen has also been detected in other tick species, e.g., *D. reticulatus*. The present assessment of the intensity of pathogen infection of ticks collected from dogs demonstrated the highest prevalence of *A. phagocytophilum*. This pathogen was detected in 8.6% (13/152) of the ticks, which confirmed the risk posed to animals. Similar rates of *A. phagocytophilum* infection were determined in female and male specimens. This is important in terms of the risk of pathogen transmission, as *D. reticulatus* males ingest small amounts of blood repeatedly to initiate spermatogenesis, which indicates that both male and female *D. reticulatus* can be involved in pathogen transmission (Bartosik et al. 2019; Földvári et al. 2016).

Table 2 Rates of infection with *Anaplasma phagocytophilum* and *Borrelia burgdorferi* s.l. in *Dermacentor reticulatus* ticks removed from dogs

		No. ticks collected	No. (%) positive for each pathogen		Total no. (%) infected ticks
			A. phagocytophilum	*B. burgdorferi* s.l.	
Ticks	Female	71	6 (8.5)	0 (0.0)	6 (8.5)
	Male	81	7 (8.6)	1 (1.2)	8 (9.9)
Dogs	Owned	74	9 (12.2)	0 (0.0)	9 (12,2)
	Stray	78	4 (5.1)	1 (1.3)	5 (6.4)
Total		152	13 (8.6)	1 (0.7)	14 (9.2)

Previous studies on *D. reticulatus* tick populations from Poland showed highly diverging percentages of infections with this pathogen. In studies on *D. reticulatus* ticks infesting dogs in urban areas of northeastern Poland, no *A. phagocytophilum* DNA was detected at all (Michalski et al. 2020). A low infection rate was also observed in ticks collected by flagging in the area of Łęczyńsko-Włodawskie Lakeland (eastern Poland), where the presence of *A. phagocytophilum* was detected in only 1.1% (7/634) of ticks (Zając et al. 2017). In turn, high numbers of ticks infected with *A. phagocytophilum* (30.4%) were reported in a study of *D. reticulatus* ticks collected from vegetation and animals in southeastern Poland. Concurrently, the study did not show the presence of *A. phagocytophilum* in any of the ticks collected from animals (including dogs) (Roczeń-Karczmarz et al. 2018). A high prevalence of *A. phagocytophilum* (32.7%) was detected in *D. reticulatus* isolated from wildlife animals (deer and roe deer) shot during hunting in some districts of Warmia-Mazury Province (north-eastern Poland) (Szczotko et al. 2019).

Low percentages or absence of *A. phagocytophilum* infections were most often observed in countries neighboring Poland. No *A. phagocytophilum* was detected in *D. reticulatus* ticks in studies conducted in Latvia (ticks collected from dogs; Naminа et al. 2019), Belarus (ticks collected from the vegetation and from cows; Reye et al. 2013), or western Siberia in Russia (ticks collected by flagging; Rar et al. 2005). In turn, the presence of *A. phagocytophilum* DNA was confirmed only in 3.6% (18/500) of ticks tested in a study conducted in the Czech Republic (ticks collected by flagging; Rybářová and Široký 2017). Different results were reported in studies of *D. reticulatus* ticks from western Ukraine, where the *A. phagocytophilum* infection rate was estimated at 15.9% (ticks collected by flagging; Ben and Lozynskyi 2019). Even higher infection prevalence was detected in a study of ticks conducted in the Chernobyl exclusion zone, which reported 25.4% prevalence of *A. phagocytophilum* infection (ticks collected by flagging; Karbowiak et al. 2014).

Borrelia burgdorferi sensu lato

In the present study, only one of the analyzed ticks (0.7%) was infected by *B. burgdorferi* s.l. spirochetes. In previous studies on *D. reticulatus* conducted in Poland, varied levels of *B. burgdorferi* s.l. infections were reported, i.e., from 0.09% (Mierzejewska et al. 2015a) to 22.8% (Roczeń-Karczmarz et al. 2018), as in the case of *A. phagocytophilum*.

Investigations conducted by Michalski et al. (2020) on ticks collected from dogs (northeastern Poland) showed a substantially higher percentage of *B. burgdorferi* s.l. infections than in the present study. The presence of *B. burgdorferi* DNA was detected in 14.1% of analyzed ticks. These results were opposite to the estimated levels of *A. phagocytophilum* infection, as the present study showed that 8.6% of the ticks were infected, whereas no DNA of the pathogen was detected by Michalski et al. (2020). An even higher *B. burgdorferi* infection rate (22.8%) was found in *D. reticulatus* collected from vegetation and animals in southeastern Poland. However, the infection rate in ticks collected from animals was just 6.7% (only three specimens of the 45 ticks collected from the animals—two ticks collected from cats and one tick from a dog were infected by *B. burgdorferi*; Roczeń-Karczmarz et al. 2018).

In studies of ticks collected by flagging in various parts of Lublin Province (eastern Poland), *Borrelia* DNA was detected in only 0.6% of *D. reticulatus* specimens (Dzięgiel et al. 2014). In other studies conducted in this province, *B. burgdorferi* s.l. infection was

detected in 1.6% of the analyzed *D. reticulatus* (Zając et al. 2017). Even lower values were reported in studies on ticks from other areas of Poland, where the prevalence of *B. burgdorferi* s.l. was estimated at 0.09% (1/1107) (Mierzejewska et al. 2015a). A low prevalence or absence of *B. burgdorferi* s.l. in *D. reticulatus* ticks was also noted in other countries. No *B. burgdorferi* s.l. spirochetes were detected in ticks in Latvia (ticks collected from dogs; Namiņa et al. 2019), Serbia (ticks collected by flagging; Tomanović et al. 2013), Germany (Richter et al. 2013), and Great Britain (ticks collected by flagging; Tijsse-Klasen et al. 2013), and their low prevalence was reported from France (1.5%) (ticks collected by flagging; Bonnet et al. 2013), Belarus (2.7%) (ticks collected from vegetation and cows; Reye et al. 2013), and western Siberia in Russia (3.6%) (ticks collected by flagging; Rar et al. 2005). As in the case of the *A. phagocytophilum* infection analyzed here, significantly higher rates of *B. burgdorferi* infection were reported in Ukraine. In studies of ticks collected by flagging in 2009–2014 in western Ukraine, the pathogen was detected in 31.9% of *D. reticulatus* (Ben and Lozynskyi 2019). Despite the higher percentages of *D. reticulatus* infections by *B. burgdorferi*, albeit rarely observed, it has been indicated that this species is unable to serve as a competent vector of *B. burgdorferi* s.l. (Grubhoffer et al. 2005). As demonstrated by Mátlová et al. (1996), in contrast to *I. ricinus* (a competent vector), a gradual decline and loss of *B. burgdorferi* s.l. was noted in *D. reticulatus* shortly after infection. Rudolf and Hubálek (2003) analyzed the impact of extracts from tick salivary glands and midguts on *B. garinii* growth in *in vitro* conditions. It was found that the extract originating from *I. ricinus* exerted a considerable stimulatory effect on the growth of the spirochetes, whereas *D. reticulatus*-derived extracts did not stimulate, but rather inhibited the *in vitro* growth of the pathogen.

Conclusions

The rapid spread of *D. reticulatus* to new territories in many European countries (including Poland) and the increase in population density in areas of their regular occurrence enhance the risk of exposure of domestic animals and their owners to tick-borne diseases. The large number of dog infestations by *D. reticulatus* ticks implies the need for studies of the prevalence of pathogens with medical and veterinary importance in order to assess the risk of tick-borne diseases. The assessment of the degree of infection of the analyzed ticks with the two pathogens revealed the highest prevalence of *A. phagocytophilum*. It is necessary to carry out further investigations of the role of *D. reticulatus* as a vector of pathogens posing a threat to humans and animals.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

No approval of research **ethics committees** was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species.

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