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Comparison of automated versus manual analysis of corneal endothelial cell density and morphology in normal and corneal endothelial dystrophy-affected dogs

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Abstract

Objective: To determine the efficacy of automated imaging software of the Nidek ConfoScan 4 confocal biomicroscope at analyzing canine corneal endothelial cell density and morphology in health and disease, by comparing to a manual analysis method.

Animal studied: Nineteen eyes of 10 dogs were evaluated and include 3 Beagles, 3 Jack Russell Terriers, and 4 miscellaneous breeds. Twelve clinically normal and seven eyes affected with corneal endothelial dystrophy (CED) were scanned and analyzed.

Procedures: Endothelial cell density (ECD), mean and standard deviation (SD) of cell area, percent polymegathism, mean and SD of the number of cell sides, and percent pleomorphism were calculated using automated and manual methods for each scan.

Results: The automated analysis showed significantly greater ECD in comparison to the manual frame method due to misidentification of cell domains in CED-affected dogs. No significant differences in ECD were observed between normal and CED-affected dogs in automated analysis, while CED-affected dogs showed significantly lower ECD in manual frame method and planimetry. Using both automated and manual methods, CED-affected dogs showed greater variability of cell area or the number of cell sides than normal dogs.

Conclusion: The automated imaging software is unable to accurately identify cell borders in CED-affected dogs resulting in inaccurate estimates of ECD. Thus, manual analysis is

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Conflicts of Interest: None

recommended for use in clinical trials assessing adverse events associated with novel medical treatments and/or surgical procedures.

Keywords

Endothelial cell density; Manual analysis; Nidek ConfoScan 4; NAVIS automated software; Dog; Corneal endothelial dystrophy

INTRODUCTION

Both specular and *in vivo* confocal microscopy have been described in dogs as non-invasive methods to assess corneal endothelial cell density (ECD) and morphology *in vivo*.[1–3] Recently, we described the advanced imaging characteristics of canine corneal endothelial dystrophy (CED), characterized by premature endothelial cell degeneration leading to concomitant corneal edema, in Boston Terriers, German Shorthaired Pointers and German Wirehaired Pointers.[4,5] Canine CED has numerous clinical, histologic, and ultrastructural similarities to Fuchs' endothelial corneal dystrophy (FECD), including corneal endothelial pleomorphism (ratio of hexagonal cells) and polymegathism (coefficients of variation of cell area) as well as a thickened Descemet's membrane.[4–7] Additionally, corneal endothelial loss also occurs in canine patients due to similar causes as humans such as aging,[7] intraocular surgical procedures,[8] glaucoma,[9] and diabetes mellitus.[10]

While cell density is a critical indicator of endothelial health in both human and canine patients, other parameters such as variation of cell size and shape may also be important in the early diagnosis of degenerative endothelial conditions.[11] The Confoscan 4 (Nidek Technologies, Gamagori, Japan) confocal biomicroscope contains fully automated analyzing software for assessing ECD, mean and standard deviation (SD) of cell area, mean and SD of the number of cell sides, percentage of cells with polymegathism, and percentage of cells with pleomorphism. These automated analytic tools, if reliable, would facilitate critical assessment of corneal endothelial health particularly in canine CED patients undergoing clinical trials for novel therapeutic compounds or surgical procedures.[4–7] However, previous studies have reported that automated endothelial cell analysis with the Confoscan 4 is unreliable because of its poor agreement with manual analysis in normal and diseased human patients.[12,13] Thus, the purpose of this study was to compare measurements of ECD and morphological parameters using manual versus automated analyzing methods with the Confoscan 4 in normal and CED-affected dogs.

2. MATERIALS AND METHODS

2. 1 Animals

All studies were approved by the Institutional Animal Care and Use Committee of the University of California-Davis (#17680, 17847, 19301, 19525) and were performed according to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. A Student's *t*-test power analysis showed that 6 eyes would allow us to detect a 10% difference in ECD between techniques with a power of 0.8 and an alpha of 0.05; previously reported ECDs using *in vivo* confocal microscopy in normal beagles or

CED-affected Boston Terriers were used for this analysis.[3,4] Data was compiled prospectively as well as retrospectively from 2 studies.[5,14] Nineteen eyes of 10 dogs (6 normal and 4 CED-affected dogs) were evaluated and included 3 Beagles, 3 Jack Russell Terriers (JRTs), and 4 miscellaneous breeds (Table 1); the right eye (OD) of one CED-affected dog was excluded since the corneal edema was too severe to visualize endothelial cells.

All dogs underwent a thorough physical examination then were sedated with acepromazine (0.01 mg/kg) and buprenorphine (0.01 mg/kg) or dexmedetomidine (3.0 μg/kg) intravenously prior to imaging. The dogs received a detailed ophthalmic examination which included digital slit lamp biomicroscopy (Imaging Module IM 900, Haag Streit, Koeniz, Switzerland), handheld slit lamp biomicroscopy (SL-15, Kowa American Corporation, Torrance, CA, USA), binocular indirect ophthalmoloscopy (Keeler Instruments Inc., Broomall, PA, USA) using a 28 D indirect lens (Volk Optical, Inc., Mentor, OH, USA), and measurement of intraocular pressure by applanation tonometry (Tonopen XL, Medtronic Solan, Jacksonville, FL, USA) following a drop of 0.5% proparacaine (Alcon Inc., Fort Worth, TX, USA) to the cornea.

2. 2 *In vivo* confocal microscopy

In vivo confocal microscopy (ConfoScan 4; Nidek Technologies) was performed with a 40×/0.75 objective lens on the central cornea of each eye as previously described.[3] One drop of 0.5% proparacaine was instilled in both eyes prior to imaging for topical anesthesia. An eye gel of 0.3% hypromellose/carbomer 980 (GenTeal® gel; Novartis Ophthalmics, Novartis Pharmaceuticals Corporation, East Hanover, NJ, USA) was placed on the tip of the objective lens as an optical coupling medium and the lens was manually advanced until the gel contacted the central surface of the cornea. Automatic, full scans were performed in all dogs without autoalignment and 350 images/scan were collected for each eye. Three images were selected for each eye using a region of interest (ROI) of 0.03 mm^2. Then, automated and manual analyses were performed by 2 examiners (AAS or TJC) each at different times.

In the automated analysis, cells within the ROI were traced by the Nidek Advanced Vision Information System (NAVIS) imaging software (Rev. 1.2, version 1.2.2, October 25th, 2007) on the ConfoScan 4 (Fig. 1) without manual cell border correction (semi-automated method). Then, ECD, mean and SD of cell area, percentage of cells with polymegathism, mean and SD of the number of cell sides, and percentage of cells with pleomorphism were calculated by the software and recorded. Manual analysis was then performed on the identical images. Cells were manually selected and counted by placing a dot on each cell within the ROI. Cells touching the borderlines of ROI were counted only along one top or bottom border and one right or left border. Cells touching the opposite 2 borders were omitted from analysis consistent with the ‘frame method’ (Fig. 1).[15] Then, the number of counted cells with the frame method was used in manual calculation for ECD. Then, the borders of each cell within the ROI were manually traced on the NAVIS imaging software. The cell area was automatically calculated by the software while the number of cell sides was manually counted by the examiner. Cells touching the border of the ROI were not analyzed. The mean and SD for cell area and the number of cell sides were manually

calculated with values of all cells within the ROI using the 'cell-border method' or 'planimetry' (Fig. 1).[12,16] Percentage of cells displaying polymegathism was defined by the coefficient of variation of cell area determined with the calculated SD for cell area being divided by the mean of cell area. To assess pleomorphism, the percentage of hexagonal cells (hexagonality) was calculated by dividing the number of cells with 6 sides (manually counted) by the total number of cells for which the number of sides was recorded. In the manual analysis, ECD was calculated with 2 different ways: the number of cells counted with frame method divided by the area of ROI, or the inverse of the mean of cell area calculated with planimetry (Fig. 1).[12,17,18]

2. 3 Statistical analysis

Three images from each eye were analyzed with automated and manual methods then values from each parameter were averaged. If values from both eyes were obtained, then those were averaged prior to statistical analysis. A one-way analysis of variance (ANOVA) was used to evaluate the effects of disease status (normal or CED-affected) and analyzing technique (manual or automated) for ECD, mean and SD of cell area, mean and SD of the number of cell sides, and percentage of cells with polymegathism and hexagonality followed by Holm-Sidak multiple comparison test as a post-hoc analysis. Unless specified otherwise, data are presented as mean ± SD.

3. RESULTS

In the frame method and planimetry, ECD was significantly greater in normal versus CED-affected dogs, respectively ($P = 0.02$ and $P = 0.03$, Fig. 2A). However, ECD did not significantly differ between normal and CED-affected dogs using the automated analysis ($P = 0.88$). In CED-affected dogs, ECD of the central cornea significantly differed when comparing automated analysis and the frame method ($P = 0.04$, Fig. 2A), however, no significant differences were observed between the automated analysis and planimetry ($P = 0.58$) or frame method and planimetry ($P = 0.59$).

With manual analysis, CED-affected dogs showed significantly greater cell area versus normal dogs (P = 0.01, Fig. 2B), while automated analysis showed no significant difference in cell area between normal and CED-affected dogs ($P = 0.76$, Fig. 2B). In normal dogs, no significant difference was identified between automated and manual analyses ($P = 0.85$); however, a trend towards a larger cell area was identified in CED-affected dogs using manual versus automated analysis ($P = 0.08$, Fig. 2B). Variability of cell area, using the calculated SD of cell area, significantly differed between automated and manual analyses in CED-affected dogs ($P = 0.02$, Fig. 2C). The CED-affected dogs showed significantly greater variability in cell area compared to normal dogs in both automated and manual analyses ($P = 0.004$ and < 0.001, respectively, Fig. 2C); the percentage of cells with polymegathism was significantly greater in CED-affected versus normal dogs using either measurement method ($P < 0.001$, Fig. 2D). Within the normal or CED-affected groups, the percentage of cells with polymegathism did not significantly differ between automated and manual analysis ($P = 0.34$ and 0.34, respectively, Fig. 2D).

Mean number of cell sides did not significantly differ by analysis technique or disease status (normal or CED-affected) ($P > 0.99$, Fig. 3A). Variability in the number of cell sides, using the SD of cell side number, was significantly greater in CED-affected versus normal dogs using automated or manual methods ($P = 0.01$ and 0.008, respectively, Fig. 3B). However, no significant differences in variability of cell side number or percentage of hexagonal cells, an indication of cellular pleomorphism, were observed between the two measurement techniques in normal and CED-affected dogs ($P > 0.66$, Figs. 3B and C). As expected, CED-affected dogs also showed a significantly lower ratio of hexagonal cells in both automated and manual analyses ($P < 0.001$, Fig. 3C). All values generated by both measurement techniques are shown in Table 2.

DISCUSSION

In addition to many genetic traits, dogs share a similar environment and common stressors with humans and are thus excellent models for spontaneous human disease.[19] We recently reported 2 spontaneous canine models of FECD as well as their utility in assessing a novel surgical treatment for bullous keratopathy.[4,5,14] As new therapies for CED are developed, clinical trials using dogs with CED will be especially useful for initial assessments of the safety and efficacy of these novel therapeutics.[20] Thus, it is critical to establish accurate methods for assessing corneal endothelial density and morphology.

Previous studies of human patients have reported the comparison of automated versus manual or semi-automated analysis for various confocal biomicroscopes.[12,13,21–23] In those studies, ECD estimates resulting from manually identifying endothelial cells of normal patients were found to be lower[21] or higher[13,22] than that found by use of automated analyzing software. Additionally, automated analyzing software was reported to significantly overestimate ECD in human patients who underwent corneal transplantation.[12,13] In some studies, planimetry was carried out as the reference method which is considered to be the most reliable.[12,17,18] Herein we compared automated and 2 manual methods (frame method and planimetry) for ECD analysis in normal and CED-affected canine eyes. The manual frame method provided significantly lower ECD values compared to automated analysis in CED-affected dogs consistent with previous human studies of patients with endothelial disease.[12,13] In the current study, a substantial difference in ECD between automated analysis or manual frame method and planimetry, which has been reported in human patients after keratoplasty[12], was not observed in CED-affected dogs. The most likely reason for this discrepancy is small sample size as well as relatively small sample area (ROI of 0.03 mm^2) required by difficulty in acquiring full-frame sheets of endothelium particularly in the CED-affected dogs. Since dog's cannot fix their eyes on a target, it is common to obtain images with only a portion containing endothelial cells. An ROI of 0.03 mm^2 was chosen in order to maximize the number of dogs to include particularly since we wanted to analyze 3 images per eye for each patient. With an ROI of 0.03 mm^2, a median (range) of 58 (35 to 76) and 28 (10 to 54) cells were counted in normal and CED-affected dogs, respectively. In the frame method and planimetry, ECD values in CED-affected dogs were significantly lower than that in normal dogs, as expected given that CED is a degenerative disorder of the endothelium. Surprisingly, no significant differences in ECD were observed between normal and CED-affected dogs using automated analysis. This was attributable to misidentification of cell

borders, particularly when the endothelial cells were large and pleomorphic in the CED-affected dogs. These large, abnormal endothelial cells were oversegmented by the software, which created additional cell-cell borders across the ROI, resulting in an artificially high ECD consistent with previous studies.[12,13,24] In planimetry, manual selection of cells prevented oversegmentation as cell borders were easily identified and traced in the CED-affected dogs. Imaging software on newer confocal biomicroscopes such as NAVIS by Nidek, IMAGEnet by Topcon, or EAT by Rhine-Tec has a manual option to modify borders falsely recognized by the software (semi-automated method).[12,24] Manual editing of the automated output was reported to give better agreement to manual cell counting in normal human subjects than the automated option.[22] Therefore, when using automated analyzing software, it is essential for the examiner to critically evaluate whether the software correctly identified the cells by closely examining the data output and correct the data with manual or semi-automated analysis if misidentification of cell junctions has occurred.

The limitations associated with automated analysis of corneal endothelial cells is not restricted to *in vivo* confocal microscopy but occurs with specular microscopy as well. Imaging software on some newer specular microscopes such as Konan NSP-9900 by Konan has a manual editing option.[25,26] The manual correction of misidentified cells can prevent overestimation of ECD in human patients with or without corneal abnormalities such as FECD or postoperative patients of Descemet stripping endothelial keratoplasty. [13,22,25,26] Whereas some previous studies reported corneal endothelial analysis by specular microscopy in dogs[7,8,27,28], they used automated or manual methods (planimetry or similar technique to frame method) and did not evaluate the difference between the two analysis techniques. Taking the findings in the present study into consideration, future studies comparing manual and automated analytic methods should be extended to specular microscopy and other corneal imaging modalities where this information is lacking.

Healthy corneal endothelium consists of approximately uniform cells with a hexagonal shape. By contrast, premature endothelial cell degeneration results in migration and cell spreading of the remaining cells to fill areas devoid of cells thus contributing to variability in their size (polymegathism) and shape (pleomorphism). In this study, automated analysis software tended to oversegment cell domains, which caused some cells to be recognized as smaller and more or less polygonal than their true size and shape. This misidentification can affect values of cell area and the number of cell sides thus masking the true tendency. In the current study, the automated analysis software underestimated variability in cell area, and did not detect a significant increase of cell area in CED-affected dogs. These data show that the automated analysis method is unreliable in analyzing mean and SD of cell area because of its poor agreement with manual methods. However, in analyzing other morphological parameters such as mean and SD of cell side number, percentage of cells with polymegathism and hexagonality, no significant differences were observed between automated and manual analyses in both normal and CED-affected dogs. Therefore, automated analysis may be useful in generating values for certain parameters in canine corneas because of the good agreement with manual morphological analysis.

In conclusion, the NAVIS automated software on the Confoscan 4 was unable to accurately identify cell borders in CED-affected dogs resulting in inaccurate estimates of ECD. While

manual analysis is more accurate for assessing corneal endothelial parameters, considerable time must be allocated to use this approach which may be resource limiting in busy research and/or clinical settings. The automated software may be useful to assess corneal ECD and morphology in normal dogs particularly those used in ocular drug and device development where the time required to complete manual analysis may be impractical. Data generated by automated analysis should be reviewed to ensure endothelial cell borders are correctly identified and manual or semi-automated analysis should be considered if misidentification of cell junctions has occurred.

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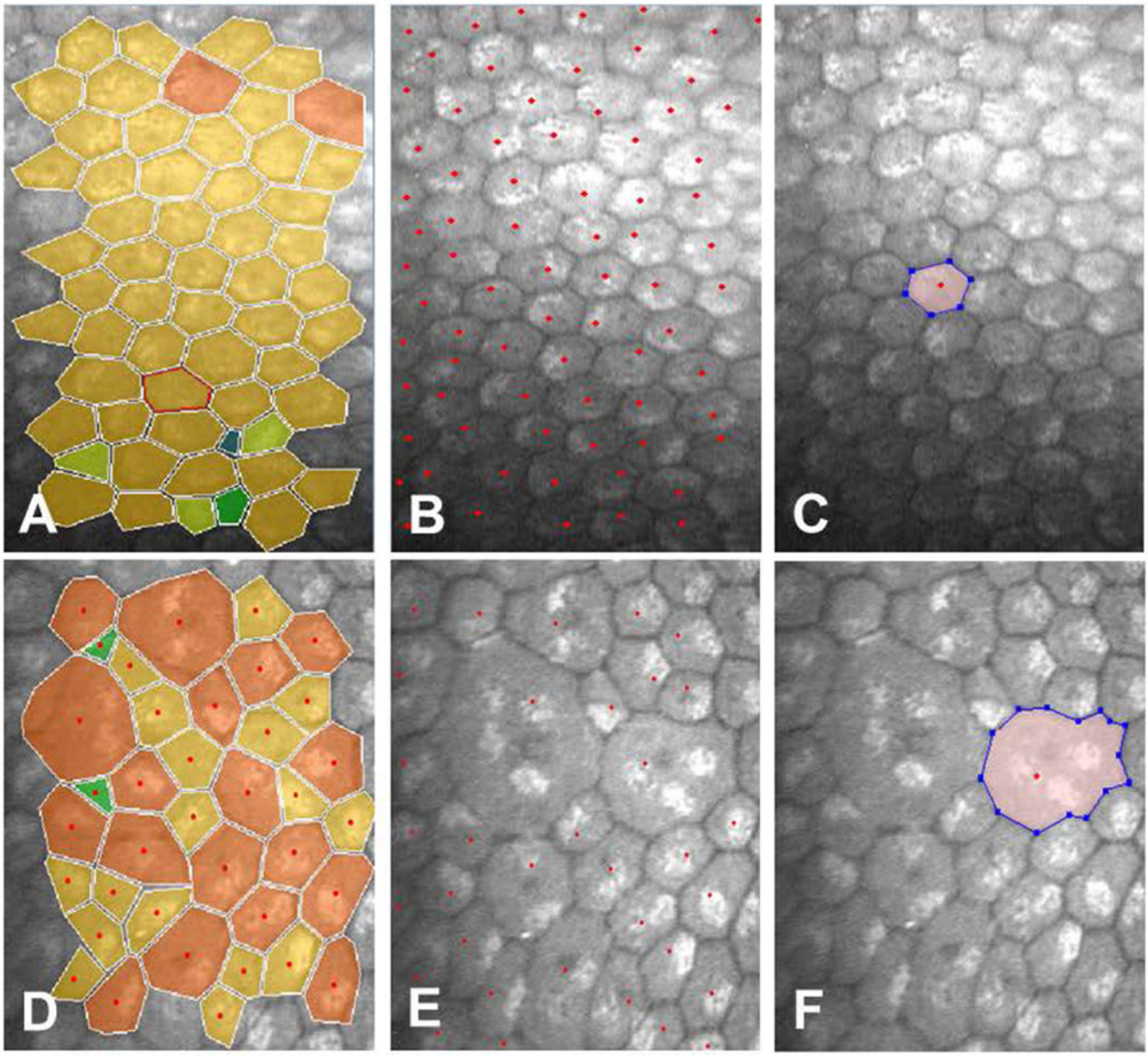

FIGURE 1. Automated analysis misidentified corneal endothelial cells in CED-affected dogs.

Top row shows representative images of the central corneal endothelium from a 5-year old, female spayed Jack Russell Terrier with a normal cornea. (A) Cells were traced and counted to be 2560 cells/mm^2 by automated analysis software, (B) estimated to be 2533 cells/mm^2 by the frame method, and (C) calculated to be 2505 cells/mm^2 from mean cell area by use of planimetry. Bottom row shows representative images of the central corneal endothelium from a 5-year old, male intact CED-affected Jack Russell Terrier with moderate pleomorphism and polymegathism and a reduced ECD. (D) Cells were traced and counted to be 1712 cells/mm^2 by automated analysis software. Note that 5 large cells were oversegmented into 18 cells. (E) The ECD was calculated to be 1133 cells/mm^2 from the

cell number counted by the frame method. (F) Cells were manually traced and planimetry was performed. The ECD was calculated to be 1113 cells/mm^2 from mean cell area.

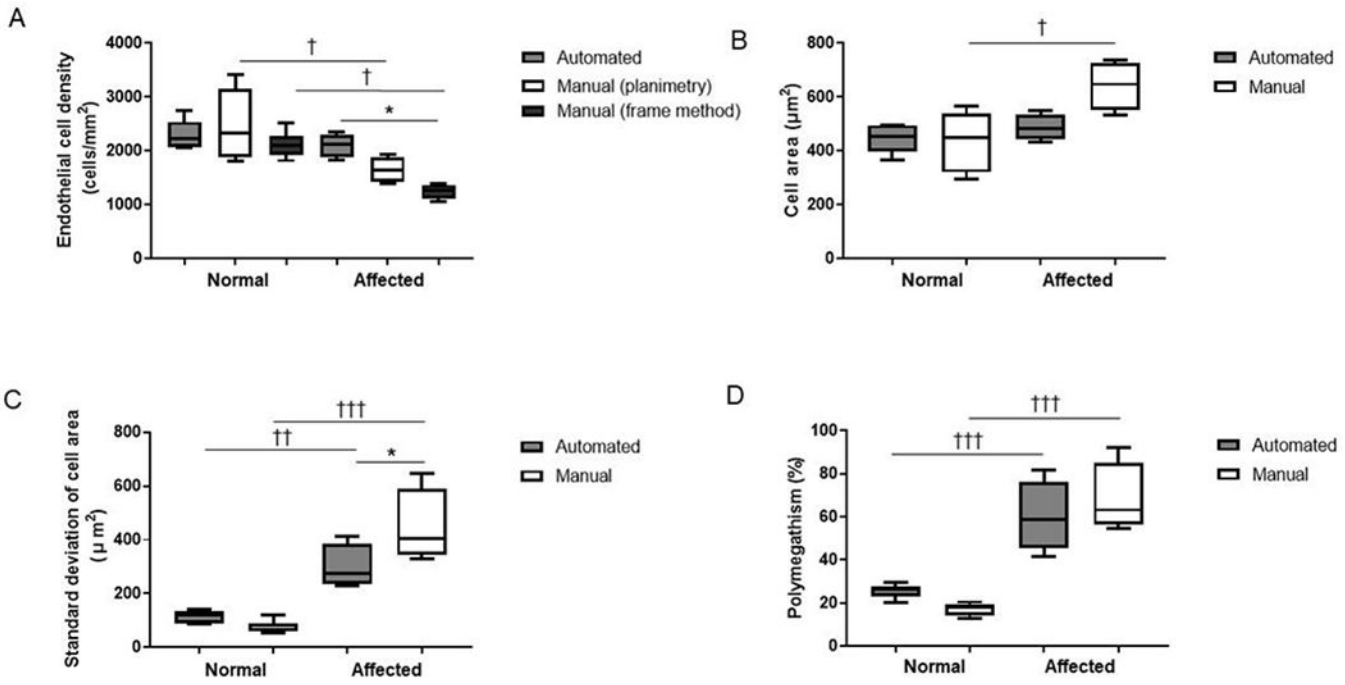

FIGURE 2. Automated analysis overestimated endothelial cell density in CED-affected dogs versus manual analysis.

(A) Automated versus manual frame analysis showed significantly higher ECD in CED-affected dogs ($P = 0.04$). The manual frame method and planimetry showed significantly greater ECD in normal versus CED-affected dogs ($P = 0.02$ and 0.03, respectively). However, automated analysis showed no significant differences of ECD between normal and CED-affected dogs ($P = 0.88$). (B) Manual analysis detected significantly larger cell area in CED-affected versus normal dogs ($P = 0.01$), however, automated analysis showed no significant difference between normal and CED-affected dogs ($P = 0.76$). (C) The SD of endothelial cell area was significantly greater for the manual versus automated analysis in CED-affected dogs ($P = 0.02$). (C, D) CED-affected dogs showed greater variability in cell area and percentage of cells with polymegathism versus normal dogs by either analysis technique ($P < 0.004$). Box plots depict median, 25th and 75th percentiles, while whiskers show minimum and maximum values. $^{*}P < 0.05$ for automated versus manual analysis; $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger}P < 0.05$ for normal versus CED-affected dogs (one-way ANOVA followed by Holm-Sidak multiple comparison test)

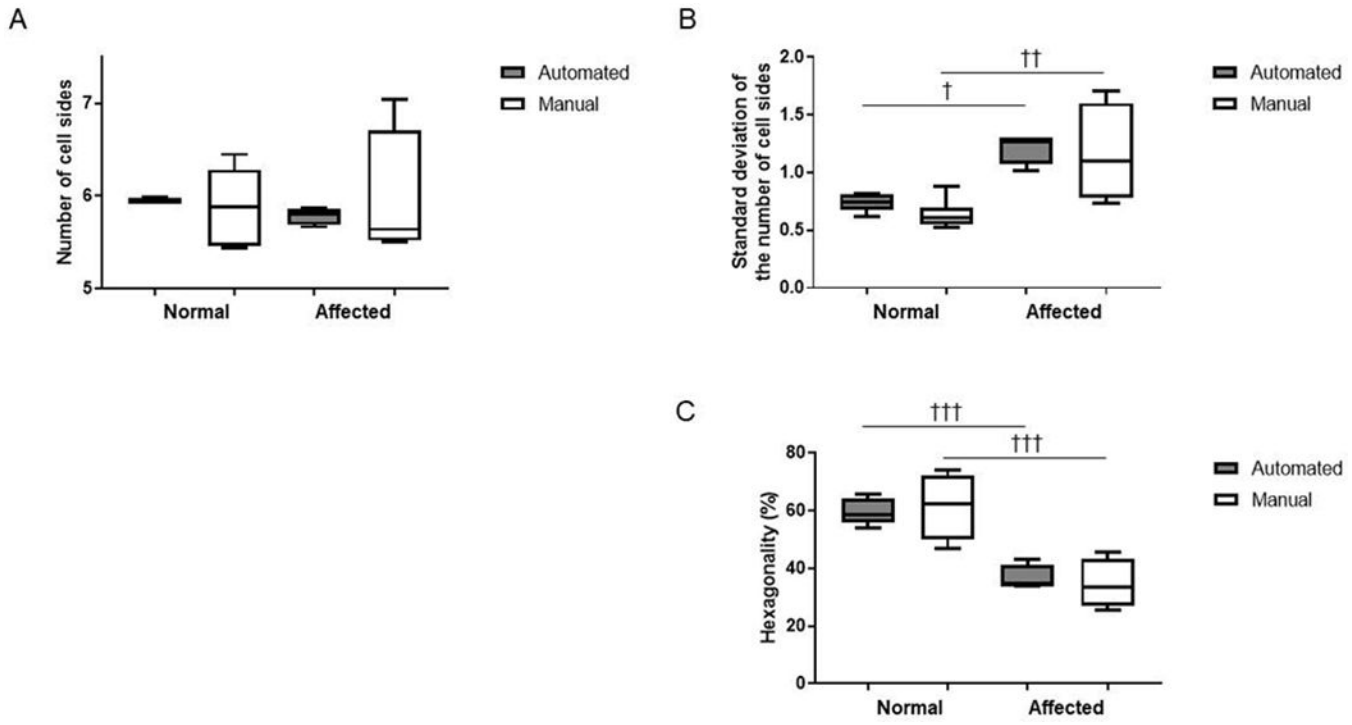

FIGURE 3. Automated analysis showed comparable values in analyzing pleomorphism to manual analysis.

(A, B, C) No significant differences were observed between automated and manual analysis in both normal and CED-affected dogs for analyzing mean and SD of cell side number, and percentage of hexagonal cells ($P > 0.66$). (B) The CED-affected dogs showed greater variability in cell side number versus normal dogs in both automated and manual analysis ($P = 0.01$ and 0.008, respectively). (C) The CED-affected dogs showed lower hexagonality versus normal dogs in both automated and manual analysis ($P < 0.001$). Box plots depict median, 25th and 75th percentiles, while whiskers show minimum and maximum values. $^{\dagger\dagger\dagger}P < 0.001$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger}P < 0.05$ for normal versus CED-affected dogs (one-way ANOVA followed by Holm-Sidak multiple comparison test)

TABLE 1.

Demographic data for study population of 10 dogs.

Age and body weight are presented as median (range).

Breed	Sex	Age (years)	Body weight (kg)	Disease state
Beagle (n = 3)	3 FI	1.8	7.3 (7.0 - 7.8)	Normal (n = 3)
JRT (n = 3)	2 FS, 1 MI	5 (5 - 7)	6.6 (6.3 - 7.2)	Normal (n = 2) CED (n = 1)
Others (n = 4)	3 FS, 1 MN	9 (8 - 11)	3.9 (2.2 - 31.1)	Normal (n = 1) CED (n = 3)

FI: female intact, FS: female spayed, MI: male intact, MN: male neutered

JRT: Jack Russell Terrier, CED: corneal endothelial dystrophy

Others: Dachshund, Pug/Chihuahua mix, Japanese chin, German Shorthaired Pointer

TABLE 2.

All calculated parameters by automated or manual analyzing methods.

Values are presented as mean ± SD.

	Endothelial cell density (cells/mm^2)	Cell area (μm^2)	SD of cell area (μm^2)	Polymegathism (%)	Cell side number	SD of cell side number	Hexagonality (%)
	Normal dogs (n = 6)						
Automated	2295 ± 278	446 ± 54	114 ± 24	25.5 ± 3.1	5.9	0.7 ± 0.1	59.5 ± 4.4
Manual (planimetry)	2480 ± 689	436 ± 120	75 ± 23	17.3 ± 2.9	5.9 ± 0.4	0.6 ± 0.1	61.4 ± 10.8
Manual (frame method)	2118 ± 247						
	CED-affected dogs (n = 4)						
Automated	2079 ± 258	489 ± 58	260 ± 31	53.0 ± 9.8	5.8 ± 0.1	1.2 ± 0.1	36.9 ± 5.4
Manual (planimetry)	1628 ± 285	655 ± 107	465 ± 165	69.5 ± 19.8	6.1 ± 0.8	1.2 ± 0.5	37.4 ± 7.5
Manual (frame method)	1314 ± 73						

CED: corneal endothelial dystrophy, SD: standard deviation