Surveillance for Transmissible Spongiform Encephalopathy in Scavengers of White-Tailed Deer Carcasses in the Chronic Wasting Disease Area of Wisconsin

Christopher S. Jennelle¹, Michael D. Samuel², Cherrie A. Nolden¹, Delwyn P. Keane³, Daniel J. Barr³, Chad Johnson⁴, Joshua P. Vanderloo⁴, Judd M. Aiken⁴, Amir N. Hamir⁵, and Edward A. Hoover⁶

¹Department of Forest and Wildlife Ecology, University of Wisconsin, Madison, Wisconsin, ²U.S. Geological Survey, Wisconsin Cooperative Wildlife Research Unit, University of Wisconsin, Madison, Wisconsin, ³University of Wisconsin, Wisconsin Veterinary Diagnostic Laboratory, Madison, Wisconsin, ⁴Department of Animal Health and Biomedical Sciences, University of Wisconsin, Madison, Wisconsin, ⁵National Animal Disease Center, ARS, USDA, Ames, Iowa, and ⁶Department of Microbiology, Immunology, and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado, USA

Chronic wasting disease (CWD), a class of neurodegenerative transmissible spongiform encephalopathies (TSE) occurring in cervids, is found in a number of states and provinces across North America. Misfolded prions, the infectious agents of CWD, are deposited in the environment via carcass remains and excreta, and pose a threat of cross-species transmission. In this study tissues were tested from 812 representative mammalian scavengers, collected in the CWD-affected area of Wisconsin, for TSE infection using the IDEXX HerdChek enzyme-linked immunosorbent assay (ELISA). Only four of the collected mammals tested positive using the ELISA, but these were negative when tested by Western blot. While our sample sizes permitted high probabilities of detecting TSE assuming 1% population prevalence in several common scavengers (93%, 87%, and 87% for raccoons, opossums, and coyotes, respectively), insufficient sample sizes for other species precluded similar conclusions. One cannot rule out successful cross-species TSE transmission to scavengers, but the results suggest that such transmission is not frequent in the CWD-affected area of Wisconsin. The need for further surveillance of scavenger species, especially those known to be susceptible to TSE (e.g., cat, American mink, raccoon), is highlighted in both a field and laboratory setting.

Transmissible spongiform encephalopathies (TSE) represent a family of infectious diseases characterized by conversion of normal prion proteins (PrP) to an abnormal form (PrP\textsuperscript{TSE}). Subsequent accumulation of misfolded prions in nervous tissues (brain and spinal cord) is associated with spongiform lesions (Prusiner, 1998), which damage and ultimately destroy cells. Invariably, TSE lead to neurodegeneration and death of infected hosts, and no treatments are currently available. Prions, and therefore TSE diseases, are generally species specific, and different strains typically afflict domestic animals, wild animals, and humans; however, birds do not appear to be susceptible to mammalian TSE (Wopfner et al., 1999). Well-known TSE include scrapie (sheep and goats; Brown & Bradley, 1998), bovine spongiform encephalopathy (BSE or “mad cow disease” in cattle; Brown, 1998), variant Creutzfeldt–Jakob disease (vCJD or human form of BSE; Will et al., 1996), transmissible mink encephalopathy (TME; Hadlow & Karstad, 1968), and chronic wasting disease (CWD in wild and captive cervids; Williams & Young, 1980). Intraspecific transmission of all of these TSE occurs through the consumption of infectious animal tissues, while scrapie and...
CWD may also be directly transmitted between infected and susceptible hosts.

Of particular concern is the potential for cross-species transmission of TSE. Though investigators demonstrated cross-species transmission through intracerebral inoculation (Bartz et al., 1998; Belay et al., 2004; Hamir et al., 2005; 2006), the potential for natural transmission by exposure to infectious TSE material in the environment is a significant ecological and public health risk. For example, it was postulated that scrapie (known for over 250 years) may have served as the origin of BSE in cattle (Wilesmith et al., 1991), which in turn induced vCJD in humans (Will et al., 1996). While there is significant evidence of transmission barriers between species (Chesebro, 2003), other studies suggest that passage of infectious materials among an intermediate host may increase the chance of TSE transmission to other species (Bartz et al., 1998). In addition, acidic conditions in the gastrointestinal tract of non-ruminant animals facilitate the denaturation of elk CWD prions and induce prion conversion, providing a mechanism for cross-species CWD transmission (Li et al., 2007).

Chronic wasting disease is a fatal condition affecting North American members of the Cervidae family (e.g., deer, elk, and moose; Williams, 2005; Baeten et al., 2007). Transmission of CWD among cervids can occur either directly (via contact between infectious and susceptible animals) or indirectly through exposure to infectious excreta, carcasses in the environment (Miller et al., 2004), blood, or saliva (Mathiason et al., 2006). Infectious prions accumulate in various tissues of infected cervids, including the alimentary lymphoid system (Sigurdson et al., 1999), central nervous system (Spraker et al., 1997), skeletal muscle (Angers et al., 2006), and cardiac muscle (Jewell et al., 2006). After host death, CWD prions from infected carcasses may persist in the soil for many years (Miller et al., 2004; Georgsson et al., 2006). Whether released into the environment through excreta or decaying carcasses, prions remain infectious (Johnson et al., 2006) and may bind with soil minerals to increase infectivity (Johnson et al., 2007). While current evidence suggests that humans are at low risk for CWD infection (Raymond et al., 2000; Belay et al., 2004), public health officials urge caution and recommend limiting human consumption of materials containing infectious prions (WHO, 2006).

Since discovery in Colorado in the 1960s, CWD was detected in approximately 12 other states and provinces across the United States and Canada (Williams & Young, 1980; Sigurdson & Aguzzi, 2007). In 2001, CWD was discovered in Wisconsin’s free-ranging deer population (Joly et al., 2003), and a subsequent investigation revealed significant heterogeneity in prevalence as a function of age and gender structure (Grear et al., 2006). While adult males in Wisconsin exhibit the highest degree of disease burden (>13% prevalence), there is considerable heterogeneity across the landscape (Joly et al., 2006). Because CWD (1) is established in wild animal populations, (2) is widely distributed geographically (Sigurdson & Aguzzi, 2007), (3) is highly resistant to environmental degradation (Taylor, 1999), and (4) crosses species barriers in experimental situations (Bartz et al., 1998; Marsh et al., 2005; Li et al., 2007), it is critically important to determine the potential for cross-species transmission in wild animals that may be exposed to infectious CWD prions. Although the risk of CWD transmission to humans is low, they can be exposed to infectious material through butchering (field-dressing) infected animals or consumption of infected meat. There may also be potential for human exposure to CWD indirectly through contact with domestic animals such as cats (Felis catus), dogs (Canis familiaris), and cows (Bos spp.), which were shown to scavenge or visit deer carcasses (Jennelle et al., 2009). Many common wildlife species may also be exposed to infectious prion materials by scavenging deer carcasses in CWD affected areas (Jennelle et al., 2009).

Given the potential exposure of wild animals to CWD from infectious deer carcasses, the goal of our study was to evaluate potential TSE infection in common mammalian scavengers in the highest CWD prevalence area of south-central Wisconsin. Our specific objectives were to: (1) evaluate the effectiveness of the IDEXX HerdChek (IDEXX Laboratories, Westbrook, ME) and Bio-Rad TeSeE CWD (Bio-Rad Laboratories, Inc., Hercules, CA) enzyme-linked immunosorbent assays (ELISA) for detecting TSEs in American mink (Mustela vison), common raccoon (Procyon lotor), domestic ferret (Mustela putorius furo), domestic sheep (Ovis aries), and striped skunk (Mephitis mephitis), (2) collect common mammalian scavengers in the CWD-affected area of Wisconsin, and (3) test these mammals for TSE infection.

METHODS

Study Area and Scavenger Collection

Our study area in south-central Wisconsin encompassed a 310-km² region characterized by the highest CWD prevalence (Joly et al., 2003): an area where CWD has been present in the environment for >20 years (Wasserberg et al., 2009). It is approximately bordered by the Wisconsin River between Sauk and Iowa Counties to the north, Ridgeway to the southwest, and Mount Horeb to the southeast. Within the study area, 812 animal carcasses, representing 8 mammalian species, were obtained from licensed trappers or as road-killed animals. Animal collection occurred over 3 years from September through April of 2003 to 2006. Necropsies were conducted on each animal, and brain tissue was excised for subsequent TSE testing using the IDEXX HerdChek ELISA procedure, which was determined to be effective in detecting the abnormal form of the prion protein in ferret, mink, raccoon, sheep, and skunk.

IDEXX HerdChek and Bio-Rad TeSeE CWD Assays

The IDEXX HerdChek Assay was used to test all mammalian tissues following the test kit procedure (IDEXX). Briefly, obex tissue was homogenized and diluted with working plate
diluent. The sample was applied to an antigen-capture plate that had a PrPSc-specific ligand immobilized on the surface. Any disease-associated conformer bound to the immobilized ligand with high affinity and the plates were washed to remove unbound materials including the normal conformer of the PrP protein. Following incubation with conditioning buffer, the captured antigen was detected using a PrP-specific antibody that was conjugated to horseradish peroxidase. The plate was washed to remove unbound conjugate, and a peroxidase substrate was added. Color development was related to the relative amounts of PrPSc captured by the ligand immobilized in the microtiter plate well and the absorbance of the microwells was read at 450 nm using a reference wavelength of 620 nm. Based on recommendations by IDEXX research personnel, samples with corrected A450–A620 values greater than or equal to the mean of the negative controls plus 0.18 were considered positive and these samples were repeated in duplicate prior to being tested by Western blot. For the serial dilution series, samples were homogenized in phosphate-buffered saline (PBS) and diluted 1:10 in PBS and treated as already described.

The Bio-Rad TeSeE assay was also employed to test samples of mammalian tissues following test kit procedures for purification and detection of PrP(CWD) (Bio-Rad). Samples of obex tissue were homogenized and purified semi-automatically using the New Sample Pr(perator) (NSP) system (Bio-Rad) in which they were treated with proteinase K; the PrPres was concentrated by precipitation and then solubilized for immunoenzymatic assay using a sandwich format. Samples were added to wells of a microplate coated with the primary monoclonal antibody. Plates were incubated, washed, and the secondary monoclonal antibody conjugated to peroxidase was added. After further incubation and washing, enzymatic activity bound to the solid phase was revealed by addition of substrate, and light absorbency of the plate was measured using a microplate reader with 450- and 620-nm filters. Bio-Rad microplate manager software was used to analyze assay results.

The effectiveness of the IDEXX HerdChek ELISA was compared to the Bio-Rad TeSeE ELISA for differentiating three TSE (CWD, scrapie, and TME) using brain and spleen tissues from American mink, common raccoon, domestic ferret, domestic sheep, and striped skunk. Ferret brain and spleen tissues from American mink, common raccoon, domestic ferret, domestic sheep, and striped skunk. Ferret brain and spleen tissues were obtained from an intracerebral (IC) challenge study that used CWD-infected mule deer brain (Sigurdson et al., 2008). Sheep tissues were obtained from a scrapie-infected flock that was also used as the source of brain material to IC challenge raccoons (Hamir et al., 2003). Raccoon TME-infected brain tissues were obtained from a study that used brain from a mink infected with TME to IC challenge raccoons (Hamir et al., 2004). Spleen tissues from TME-infected mink and skunk were obtained from J. Aiken (University of Wisconsin, Madison), and confirmed using Western blot. Forty-six samples from 23 animals were analyzed using IDEXX HerdChek and 18 samples from 9 animals using Bio-Rad TeSeE test procedures. All animals used for Bio-Rad TeSeE testing were a subset of the animals used for IDEXX HerdChek testing.

### Western Blot Testing

Samples testing positive in IDEXX HerdChek were subsequently tested for proteinase K (PK)-resistant PrP using Western blot. Prior to Western blot analysis, 100 µl of each homogenate was mixed with 100 µl 4% sarcosine in PBS followed by PK treatment (50 µg/ml for 30 min) and stopped by the addition of 1.5 µl phenylmethylsulfonyl fluoride saturated ethanol. Samples were then treated with 12.4 µl 4% phosphotungstic acid solution (pH 7.6) and incubated overnight on a 37°C rocker incubator. Insoluble PrP aggregates were enriched by centrifugation for 30 min at 12,000 × g, supernatant discarded, and the pellet was resuspended in 20 µl of a 1:1 mixture of PBS and 2× protein sample buffer. Samples were boiled 10 min following by a 1-min sonication and 5-min boil. Ten microliters of sample was loaded on a 15-well 12% bis-Tris NuPage gel (Invitrogen, Carlsbad, CA). Proteins were resolved and transferred to a polyvinylidene fluoride membrane, which was blocked with 5% milk Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 h, and PrP specific monoclonal antibodies added in milk TBST (1:20,000 6H4 monoclonal antibody [Prionics, Lelystad, Switzerland], 1:5,000 99/97.6.1, and 1:20,000 3F4). The blot was washed in TBST 3 times for 5 min, and secondary goat anti-mouse immunoglobulin (Ig) G horseradish peroxidase-conjugated antibody (BioRad) 1:20,000 in 5% milk TBST was added for 1 h at room temperature. The blot was rinsed 3 times for 5 min in TBST and the blot was visualized with Supersignal (Pierce, Rockford, IL).

### Data Analysis

Since no confirmed CWD-positive animals were found, formal statistical comparisons were not conducted. Numbers of animals sampled for TSE disease are reported by scavenger species, year of collection (September through April of 2003 to 2006), and age (juvenile versus adult). For each scavenger species collected, the probability was calculated for detecting at least a single infected individual assuming a 1% TSE prevalence occurred in the population using the formula presented in Cannon and Row (1982) and Gu and Novak (2004). For these calculations, it was assumed that test sensitivity and specificity were 100% and collected animals were randomly sampled within the CWD-affected area of southern Wisconsin. It was also assumed that the population of each scavenger species was sufficiently large that it was unaffected by our collections, resulting in a conservative estimate of TSE detection probability.

### RESULTS

#### IDEXX HerdChek and Bio-Rad TeSeE Testing

All 23 samples tested using IDEXX HerdChek correctly classified positive CWD, scrapie, and TME, as well as control
TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHY SURVEILLANCE

Comparison of IDEXX HerdChek and Bio-Rad TeSeE Test Results for Identifying Infectious Prions Associated with Transmissible Mink Encephalopathy (TME), Scrapie (SCR), and Chronic Wasting Disease (CWD), as well as a Negative Control (Uninfected)

<table>
<thead>
<tr>
<th>Disease</th>
<th>IDEXX</th>
<th>Bio-Rad</th>
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<tbody>
<tr>
<td>TME</td>
<td>Mink</td>
<td>Brain: 1/1</td>
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<tr>
<td></td>
<td>Skunk</td>
<td>Brain: 1/1</td>
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<tr>
<td>SCR</td>
<td>Raccoon</td>
<td>2/2</td>
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<tr>
<td>CWD</td>
<td>Ferret</td>
<td>4/4</td>
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<tr>
<td>Uninfected</td>
<td>Ferret</td>
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<tr>
<td></td>
<td>Sheep</td>
<td>4/4</td>
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</tbody>
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Note. Infected or uninfected brain or spleen tissue from mink, skunk, raccoon, sheep, or ferrets were used. Counts indicate the number of correctly classified individuals per number tested, and cells with an asterisk indicate a misclassification.

To further explore the sensitivity of IDEXX HerdChek to TSE agents in various host species, several TSE strains from infected animals were tested following 10-fold serial dilutions of brain homogenate (Table 2). IDEXX HerdChek demonstrated sensitive and specific identification of positive samples, even on the drowsy strain of hamster adapted TME, which is more PK sensitive than the others strains tested. When compared to Western blot, IDEXX HerdChek demonstrated 10-fold greater sensitivity to TSE strains tested with the same inoculum (data not shown).

These IDEXX HerdChek suspect-positive animals were further tested by Western blot to validate the presence of a TSE agent. All the suspect animals tested negative by Western blot (test results not shown), indicating that no TSE infections were confirmed in the animals sampled. Thus, evidence indicated the IDEXX HerdChek test scores in these cases indicated false positive results.

Of the adult animals collected, the probability of detecting at least 1 TSE-infected animal from 259 raccoons, 202 opossums, and 200 coyotes (assuming underlying prevalence of 1%) was estimated as 93%, 87%, and 87%, respectively. Due to small sample sizes (<26) obtained from the other 5 species collected (see Table 3), the probability of detecting at least 1 TSE-infected animal from these groups (assuming underlying prevalence of 1%) was at most 22%.

DISCUSSION

IDEXX HerdChek and Bio-Rad TeSeE Sensitivity

Our comparison of the IDEXX HerdChek and Bio-Rad TeSeE tests suggest that the IDEXX HerdChek is sufficiently sensitive to detect several TSE (CWD, scrapie, and TME) in different host animal species (mink, skunk, raccoon, and ferret), while the Bio-Rad TeSeE test lacks the sensitivity to detect (at least) TME in spleen (Table 1). Since the Bio-Rad TeSeE test is composed of a two-antibody system (one for

| TABLE 2 | Relative Sensitivity of IDEXX HerdChek to Diverse Strains of TSE Agent |
|---------|----------------------|------------------|----------------|-----------------|----------------|
| Dilution| Brain homogenate: 1:10 | 1:100 | 1:100 | 1:100 | 1:100 |
|         | 20% hamster hyper* | 2/2 | 2/2 | 2/2 | 1/2 | 0/2 |
|         | 20% hamster drowsy* | 2/2 | 2/2 | 2/2 | 0/2 | 0/2 |
|         | 20% white-tailed deer | 2/2 | 2/2 | 0/2 | 0/2 | 0/2 |
|         | 20% mink | 2/2 | 2/2 | 2/2 | 0/2 | 0/2 |
|         | 10% mouse RML* | 2/2 | 2/2 | 2/2 | 2/2 | 0/2 |
|         | 10% sheep | 2/2 | 2/2 | 2/2 | 0/2 | 0/2 |
|         | 20% hamster uninfected | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |
|         | 20% mouse uninfected | 0/2 | 0/2 | 0/2 | 0/2 | 0/2 |

Note. Ten-fold serial dilutions of TSE agent were made from brain homogenates of sheep (10%), hamster (hyper 20% and drowsy 20% strains), deer 20%, mink 20%, and mouse 10% (RML strain). All brain homogenates are reported as the number of positive test results per number of trials in PBS.

*Strains obtained from end-stage animals experimentally inoculated and maintained at the University of Wisconsin, Madison.

*CWD agent from a white-tailed deer harvested in the endemic area of south-central Wisconsin.

*Scrapie agent used was a pooled 10% brain homogenate from clinical sheep; provided by A. Jenny.
capture and one for detection of TSE antibodies), it is likely that one or both antibodies did not react with mustelid tissue samples. Due to the proprietary nature of the Bio-Rad test, information on specific antibodies was not available for further testing. Because our sample of reference tissues was small, one cannot make strong conclusions about the true differences between these tests, but the results suggest that Bio-Rad TeSeE may have limitations when testing for TME. Evaluation of IDEXX HerdChek using serial dilutions of TSE agents (Table 2) further demonstrated that the test was sensitive across a broad range of TSE strains and host species.

Scavenger Sampling and TSE Detection

It was not possible to document TSE infections in any of the 812 mammalian scavengers tested in the CWD-affected area of Wisconsin. Four animals tested positive using the IDEXX HerdChek test, but these were considered to be false positives based on subsequent Western blot results. These false positives may have resulted from the sensitivity of IDEXX HerdChek to bacteria or other contaminants contained in these samples, rather than detection of normal cellular PrP. It is also noteworthy that a negative result in Western blot analysis does not guarantee the absence of TSE agent. Although comparable sensitivity was achieved with the IDEXX HerdChek ELISA (data not shown), TSE detection with Western blot depends on PK resistance of the disease-associated PrP and not all TSE strains are highly PK resistant (Bessen & Marsh, 1994). Our results indicate that the IDEXX HerdChek ELISA was a valuable method for screening a large number of tissue samples for TSE in a variety of mammalian species.

For raccoon, opossum, and coyote, sample sizes of adult animals provided high probability (93%, 87%, and 87%, respectively) of detecting disease if it occurred at 1% prevalence or higher, while sample sizes of other scavengers were insufficient to provide meaningful information about the likelihood of TSE detection (Table 3). Raccoon and opossum in particular are two of the most active scavenging vertebrates of deer carcasses in south-central Wisconsin (Jennelle et al., 2009). The absence of TSE infection in these common scavengers suggests that infectious CWD prions (e.g., from deer carcass remains) have not produced frequent cross-species transmission through a natural route (e.g., consumption of infectious materials) in Wisconsin. However, our test results have several
exposure will lead to cross-species transmission, and the probability that prions from deer carcasses are transmitted to scavengers is uncertain. Successful cross-species transmission requires that (1) the rate of false negative results in our samples is unknown (although sensitivity in our trials for raccoons appeared to be 100%; Table 1), (2) the ability of the IDEXX HerdChek ELISA to detect a TSE is limited by prion concentration in the sample (i.e., which likely correlates with how long an animal has been infected) (Table 2), (3) the age of sampled scavengers was qualitatively classified but one cannot be certain of whether animals were old enough to show detectable levels of TSE if present, and (4) TSE-positive tissues (as test controls) were lacking for most scavenger species tested. Although felines are susceptible to TSE (Pearson et al., 1992), feline samples for validation of IDEXX HerdChek were not available. Furthermore, badger, canid, and opossum samples were not validated, as it was not possible to find published information showing that animals in these groups can be naturally or experimentally infected with a TSE. Overall, data showed IDEXX HerdChek broadly applicable to a variety of host species, useful to screen large numbers of wild animals in a short period of time, and it is recommended for further validation studies for other species and TSE.

For species that are known to be susceptible to TSE diseases (mink and cats) our sample sizes were insufficient to detect meaningful levels of prevalence. While some studies found that raccoons artificially inoculated with the mule deer (Odocoileus hemionus) strain of CWD were not adversely affected (Hamir et al., 2003), one cannot completely rule out the possibility that a successful cross-species CWD transmission has occurred in nature. A further complication for CWD is that cervid PrP genotypes have been linked to disease susceptibility (O'Rourke et al., 2004; Jewell et al., 2005; Johnson et al., 2006) or rate of progression (Keane et al., 2008), but apparently do not present a major barrier to the spread of CWD among cervids. Although CWD has likely been present in our study area for >20 years (Wasserberg et al., 2009), there is considerable uncertainty about the rates of scavenger exposure to infectious prions from deer carcasses, the probability that exposure will lead to cross-species transmission, and the postinfection period required to produce detectable prions in scavenger brain tissue. Successful cross-species transmission typically results in extended incubation periods in new hosts (Kimberlin, 1979; Kimberlin et al., 1978), which may preclude short-lived scavenger species, juvenile animals, or younger adults from having detectable levels of TSE. After initial passage of a TSE in a new host species, however, incubation periods shorten and consistent patterns in disease progression emerge (Kimberlin & Walker, 1977, 1978).

Future laboratory challenge studies are needed, and field testing, of scavenger species that are common consumers of deer carcasses (Jennelle et al., 2009) in southern Wisconsin. Field sampling efforts need to focus particularly on adult age classes of longer lived species (as they would be exposed to TSE agent for a longer period of time), while laboratory challenges would be better suited for young animals due to the typically long incubation time of TSE diseases. Because TSE occur in cats, mink, raccoons, and skunks (by natural or unnatural routes; Eckroade et al., 1973; Pearson et al., 1992; Kirkwood & Cunningham, 1994; Hamir et al., 2003), future research needs to focus on these species (or related species) for future scavenger TSE screening. Although opossum, coyote, and red fox can be common scavengers at deer carcasses (Jennelle et al., 2009), little is known about their risk of TSE infection, which warrants further attention.

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